ANALYSIS OF A MODEL TO DESCRIBE LEAD TRANSPORT BY THE SMALL INTESTINE

A Thesis submitted for the degree of

Doctor of Philosophy

by

MAURICE JOHN COOGAN

Department of Chemistry

The University of Aston in Birmingham

June, 1982

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SUMMARY

Investigations into the mechanism of lead transport across the rat small intestine were conducted using the in vitro everted sac technique. The data obtained were used to construct a model which could competently rationalise some of the reported observations in the literature concerning intestinal lead transport.

The model comprises of two individual processes acting in concert. A large rapid adsorption of lead onto the intestinal tissue surface; and a concurrent slow passive diffusion of lead cations across the intestinal epithelium.

The presence of methylxanthines, which diminish the size of the intercellular channels, were found to decrease the rate of lead cation transport, and indicated a paracellular route for lead transport. Certain metal cations compete with lead for transport via the paracellular route and hence decrease its rate of transit. Other metal cations increase the tissue binding of lead by a process of coprecipitation, and may ultimately increase the entry of lead into the body.

Chelated lead species exhibit different kinetic characteristics to that of the lead cation. The data suggests that chelation accelerates lead absorption, probably by the utilisation of a transcellular transport route.

The studies indicate why, under noraml conditions, only a small percentage of lead is absorbed, and also suggest the manner by which larger amounts of lead may gain entry into the body.

KEY WORDS

Intestinal Lead Absorption

Paracellular Transport

Adsorption, Coprecipitation

DECLARATION

This thesis is an account of original work carried out in the Department of Chemistry of the University of Aston in Birmingham, between February, 1979 and January, 1982. This work has been carried out independently and has not been submitted for any other degree.

Maurice J. Coogan

Maurice John Coogan

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to all those people involved in the preparation and presentation of this thesis, for encouragement, discussion, and general advice.

I would particularly cite Professor J. A. Blair and Dr. M. E. Hilburn for their supervision and support.

I am indebted to the staff of the Department of Clinical Biochemistry and of Histology of the Birmingham General Hospital for the use of facilities there.

I dedicate this thesis to my parents

Maurice and Florence Helen, and to

my brother Michael Allen, and sisters

Catherine Anne, Gillian Margaret

and Susan Shirley

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CHAPTER ONE

INTRODUCTION

1.1. PATHOLOGICAL EFFECTS OF LEAD

Lead has been recognised for centuries as a general metabolic poison, the main target organs are the kidneys and liver. Lead is accumulated in the soft tissues and particularly by the skeleton, (Waldron and Stofen, 1974). Lead poisoning results in a well-characterised syndrome manifested in adults as colic, anaemia, headache, fatigue, peripheral neuropathy and hypertension, (Hamilton et al, 1949). Such symptoms of lead poisoning in adults, it was thought, usually do not occur until blood lead levels exceed 120 $\mu g\ dl^{-1}$, but more recently this figure has been reduced to 80 $\mu g\ dl^{-1}$ (Damstra, 1977), and a D.H.S.S. working party (D.H.S.S., 1980) has suggested that levels greater than 35 $\mu g\ dl^{-1}$ should be investigated.

In children the symptoms of acute lead intoxication include vomiting, anorexia, lethargy, convulsions, coma and encephalopathy, (Hardy, 1971). Controversy exists, however, regarding the possible neurological effects of continuous exposure to lower levels of lead, (David et al., 1976 and Needleman et al, 1979). There is no general agreement on the concentration of blood lead that could be considered dangerous. Albert et al., (1974) and Sachs (1974) noted definite signs of neurological damage in children with blood lead levels of just over 60 $\mu g\ dl^{-1}$. However, more recent investigations in school children has suggested that there is a significant association between much lower blood lead levels (e.g. 26 $\mu g\ l^{-1}$) and impaired

intelligence, (Yule et al, 1981).

1.2. THE MOBILISATION OF LEAD

The presence of lead in the earth's crust results in the natural mobilisation of the metal into the environment. As a consequence of its presence in the soil (16 ppm., Stubbs, 1972) and natural bodies of water (0.001 to 0.01 ppm., Stubbs, 1973), lead enters the food chain and becomes incorporated into plant and animal tissues. The advent of the Industrial Revolution and the subsequent growth of industry has increased the use and mobilisation of lead, (Table 1-1). The modern practice of adding lead to petrol has added considerably to the burden of lead in the environment, and constitutes the major source of lead in the atmosphere of cities, (Waldron and Stöfen, 1974).

Much attention has recently been paid to the question of the relative contribution made to the body burden of lead by airborne and dietary lead. It is the conclusion of a D.H.S.S. working party on lead in the environment, (D.H.S.S., 1980) that adults who are not specifically exposed to lead derive 45-90% of their blood lead from food, 0-45% from water and 10-20% from air. A report prepared for the Conservation Society (1980) suggests that the value quoted for the daily lead intake from water reported by the D.H.S.S. working party is exaggerated, and the contribution made by airborne lead to blood lead levels is a more significant proportion. However, the Conservation Society report (1980) does not take into account

TABLE 1.1. WORLD REFINED CONSUMPTION OF LEAD
(THOUSAND METRIC TONS)

YEAR	U.K.	EUROPE	U.S.A.	WORLD TOTAL
1940	348	747	563	1653
1945	240	374	704	1325
1950	236	640	803	1856
1955	285	880	735	2262
1960	286	1061	647	2633
1965	312	1197	753	3179
1970	262	1358	826	3805
1975	238	1169	811	3878
1980	295	1637	1094	5290

U.K. USES OF LEAD (THOUSAND METRIC TONS

	1951	1961	1971	1980
Batteries	62	68	101	98
Tetraethyl	5	25	44	63
Sheet and pipe	72	73	54	51
Cables	92	97	52	21
Oxide and other compounds	34	23	31	18
Alloys	15	21	17	21
Solder	12	16	14	-
Shot	6	6	6	7
White lead	21	8	3	0.9
Collapsible tube	6	4	2	1.4
Rolled & extruded products	-	7	2	0.4
Miscellaneous	17	20	20	21
TOTAL	341	369	346	296

Taken from "World Metal Statistics"

adventitious sources of lead, principally from paint, dust, soil and certain cooking utensils, that would add greatly to the lead present in the diet, and totally outweigh any contribution to blood lead values made by airborne lead. Thus the principal route for lead entry into the human body is by the gastrointestinal tract. In order to determine and understand the possible mechanism of lead transport across the small intestine a knowledge of intestinal morphology is necessary.

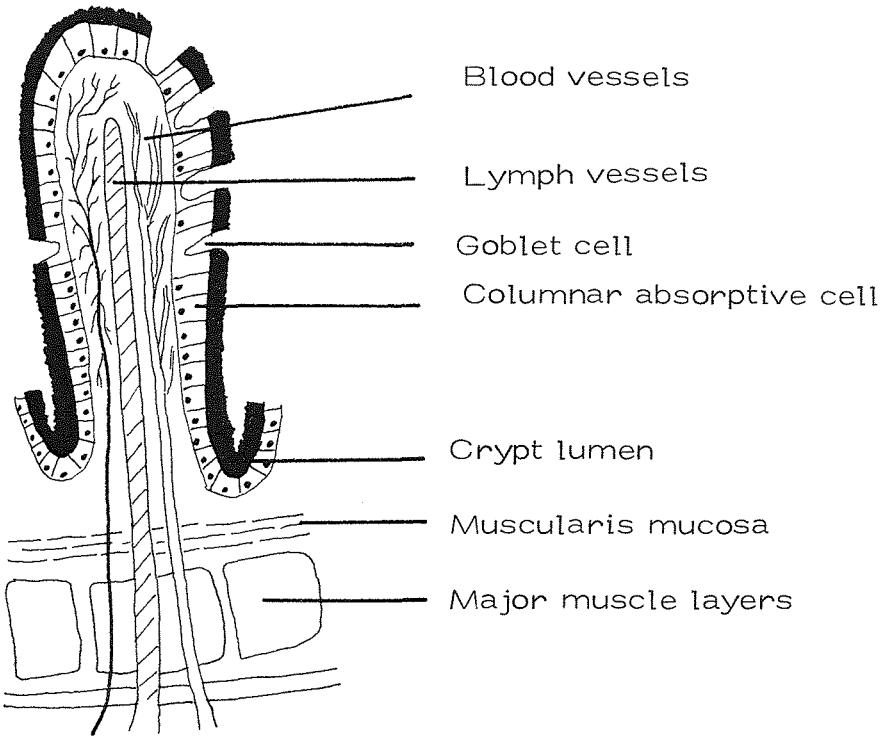
1.3. THE MORPHOLOGY OF THE SMALL INTESTINE

The small intestine is essentially comprised of three layers; the epithelium, the lamina propria and the muscularis mucosae.

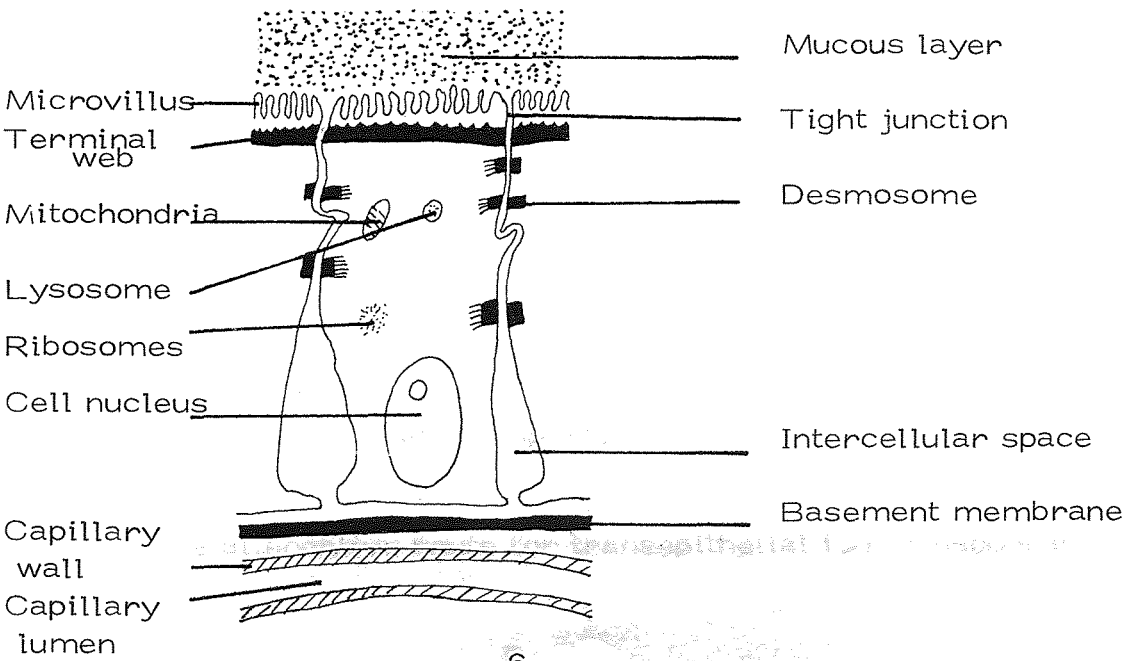
The intestinal epithelial mucosa is a one-cell thick uniform layer which is composed primarily of columnar epithelial cells, most of which are absorptive cells. Other cells include goblet, basally and apically granulated, and vacuolated, which compared to the absorptive cells are relatively few in number and make little contribution as a transport pathway. Absorptive cells originate in the crypts and migrate, depending upon the animal species, over a few days to the tips of the villi, where they slough off into the lumen, (Figure 1-1a). The absorptive capacity of cells at the base of the villus is thought to be less than that of mature cells, while cells at the villus tips are considered to be senscent and also of a low absorptive capacity.

Figure 1.1

a) A schematic diagram of a sectional villus



b) A schematic diagram of an intestinal columnar absorptive cell



The epithelium and lamina propria form villi which are finger-like projections about 1 mm in height that protrude into the intestinal lumen and crypts. The lamina propria contains the blood and lymphatic vessels that supply nutrients and remove absorbed materials from the villi.

The muscularis mucosae is a thin layer of involuntary muscle through which the blood and lymphaticvessels pass into the submucous region, (a region of circular and longitudinal muscles).

Possible routes of transport across the intestinal epithelium

The intestinal epithelial cells are joined at their apical surfaces to form the tight junctions, (Figure 1-1b). Immediately beneath the tight junctions are spaces, (lateral intercellular spaces) bounded by the cell walls and basement membrane. The intercellular spaces are relatively small just below the tight junctions, but increase in size towards the basement membrane, (Spring and Hope, 1978). The intestinal epithelium is an example of a leaky epithelium and it is thought that water transport, induced either hydrostatically or osmotically, occurs, to some extent, via the tight junctions and intercellular spaces, i.e. via a paracellular pathway. The paracellular pathway may also be important for lipophilic non-electrolyte transepithelial transport.

The alternative route for transepithelial ion transport is

across the cell membrane, i.e. via a transcellular pathway. Transport of species via this route from the lumen will result in crossing various boundaries, each of which may in themselves constitute a barrier to transport. Some of the more important potential barriers are mentioned briefly.

The aqueous stagnant layer - The thickness of this "unstirred" layer has been estimated *in vivo* to be 530 μm (Winne, 1976) and *in vitro* to range between 150 to 200 μm, (Sallee and Dietschy 1973 and Wilson and Dietschy, 1974). It is believed that the aqueous stagnant layer is an effective barrier for both the paracellular and transcellular transport of species that penetrate relatively rapidly.

The mucous layer - This can be considered to be a functional component of the unstirred layer. The mucous layer is secreted by the numerous goblet cells. Rat goblet cell mucin is a glycoprotein (molecular weight 2×10^6) and is negatively charged. It is composed of hexosamine, protein, sialic acid and fucose. It has been reported that the gastric mucous barrier prevents rapid diffusion of hydrogen ions, (Pfeiffer, 1981). Mucin also reduces permeability to positively charged species, (Kellaway and Marriot, 1975) and quarternary amines, (Levine *et al.*, 1955). The permeability reduction of these species is thought to be due to binding by the negatively charged mucin. The mucous layer is also a potential barrier to both paracellular and transcellular routes of transport

across the epithelium.

The apical membrane - Generally, the apical membrane structure is very similar to other biological membranes, consisting of a bilayer of lipid with protein embedded in, or extending across the lipid. The protein components may provide a polar pathway for poorly lipid soluble compounds to traverse the membrane.

The membrane is in the form of microvilli that cover the luminal surface of the absorptive cell. The apical membrane surface is increased by an estimated factor of twenty by the microvilli, compared to the same surface without these projections. On average the microvilli are 1 μm long and approximately 0.1 μm wide, and are separated from one another by narrow channels approximately 50 nm wide. The microvilli are covered by a thin, loose meshwork of mucopolysaccharide filaments, referred to as the glycocalyx, which is approximately 0.1 to 0.5 μm thick and may serve to anchor the mucous layer. It is also believed that the glycocalyx also serves to maintain a region of low acidity between the apical cell membrane and the luminal contents, known as the acid microclimate, (Blair and Matty, 1974). The transport of certain charged species, in particular, organic acids such as folic acid, may be facilitated by such a region, (Lucas, 1979).

The epithelial cell contents - Transcellular diffusion

may be affected by various intercellular organelles, such as the cell nucleus, the endoplasmic reticulum, the Golgi material, the mitochondria and lysosomes. It is assumed that no concentration gradient exists across the cell, and it has been suggested that the cytoplasm and the cell fluid may have similar permeability properties to apical and basal cell membranes.

The basal and basement membranes - The basal cell membrane is a 7-9 nm thick bilayer membrane with permeability properties similar to those of the biological membranes previously described. The basal membrane lies close to the basement membrane, and there may be a relatively narrow water filled extracellular space between them.

1.4. FACTORS THAT AFFECT THE TRANSPORT OF LEAD

Table 1.2 displays some of the factors that have been reported to affect the absorption or retention of lead. Much of the data has been obtained from animal feeding experimentation, but there has been little rationalisation of the observations by mechanistic models of lead transport. High dietary doses of calcium has been demonstrated to decrease lead absorption, (Barton et al., 1978, Gruden, et al., 1974), and Meredith et al., 1977), and low dietary calcium to enhance lead absorption, (Mahaffey et al., 1973, Meredith et al., 1977, Six and Goyer, 1972 and Barltrop and Khoo, 1976). Iron

TABLE 1.2. FACTORS THAT AFFECT LEAD ABSORPTION

FACTOR	EFFECT	AUTHOR
MILK		
(Consumption)	+	Kello and Kostial (1973)
	O	Garber and Wei (1974)
	+	Stephens and Waldron (1975)
	+	Bell and Spickett (1981)
METALS		
Calcium		
(low)	+	Ledered and Bing (1940)
(low)	+	Mahaffey et al., (1978)
(low)	+	Quarterman and Morrison (1975)
(low)	-	Gerber and Deroo (1975)
(low)	+	Barltrop and Khoo (1976)
(high)	-	Barltrop and Khoo (1976)
(deficiency)	+	Mykkänen and Wasserman (1981)
Magnesium		
(high)	-	Fine et al., (1976)
Zinc		
(low)	-	Cerklewski and Forbes (1976)

TABLE 1.2. (continued....)

FACTOR	EFFECT	AUTHOR
Phosphorous		
(low)	+	Quarterman and Morrison (1975)
(low)	+	Barltrop and Khoo (1976)
(high)	–	Barltrop and Khoo (1976)
Iron		
Deficiency	+	Six et al.,(1972)
Deficiency	+	Ragan (1977)
Deficiency	+	Barton et al., (1978b)
Loaded	–	Barton et al., (1978b)
Deficiency	+	Hamilton (1978)
Deficiency	+	Flanagan et al., (1979)
Deficiency	+	Watson et al., (1980)
Loaded	–	Barton et al., (1981)
CHELATING AGENTS		
Amino acids (with sulphydryl groups)	+	Conrad and Barton (1978)
Nitrilotriacetic acid	+	Garber and Wei (1974)
Sodium citrate	+	Garber and Wei (1974)

Continued..

TABLE 1.2. (continued....)

FACTOR	EFFECT	AUTHOR
Sodium citrate	+	Jugo et al., (1975)
Citric acid	+	Garber and Wei (1974)
Ascorbic acid	+	Conrad and Barton (1978)
Ethylenediaminetetraacetic acid	O	Garber and Wei (1974)
Ethylenediaminetetraacetic acid	+	Jugo et al., (1975)
Ethylenediaminetetraacetic acid	O	Garber and Wei (1974)
2,3 Dimercaptoproponol	+	Jugo et al., (1975)
D-penicillamine	+	Jugo et al., (1975)
Lecithin	+	Quarterman et al., (1977)
Choline	+	Quarterman et al., (1977)
Pectate	-	Paskins-Hurlburt et al., (1977)
Fat	+	Quarterman et al., (1977)
Fat	+	Barltrop and Khoo (1976)
OTHER DIETARY FACTORS		
Alcohol	-	Barton and Conrad (1978)
Bile secretion	+	Cikrt and Tichy (1975)
Bile salts	+	Quarterman et al., (1977)
Vitamin D	+	Smith et al., (1978)
Corn oil	+	Barltrop and Khoo (1976)

TABLE 1.2 (continued....)

FACTOR	EFFECT	AUTHOR
MISCELLANEOUS FACTORS		
Fasting	+	Wetherill et al., (1975)
Starvation	-	Conrad and Barton (1978)
Starvation	+	Chamberlain et al., (1978)
Food restriction	+	Quarterman et al., (1976)
Absence of food	+	Rabinowitz et al., (1980)
The presence of food	-	Garber and Wei (1974)
Fasting	+	Hilburn et al., (1981)
AGE		
(young)	+	Kostial et al., (1971)
(young)	+	Forbes and Reina (1972)
(young)	+	Gerber and Deroo (1975)
PARTICLE SIZE		
(small)	+	Barltrop and Meek (1979)
PINOCYTOSIS	+	Keller and Doherty (1980)

KEY			
	+	=	Increased lead absorption
	-	=	Decreased lead absorption
	O	=	No effect on lead absorption

deficiency has been reported to increase the body retention of lead, (Six and Goyer, 1972), and excass dietary zinc (Cerklewski and Forbes, 1976) or magnesium (Fine et al., 1976) has been demonstrated to decrease lead absorption. It is from such data that the present mechanistic models to describe lead transport have evolved. Other dietary factors reported to alter lead absorption are protein and fat constituents of the diet (Kello and Kostial, 1973) and chelating agents (Jugo et al., 1975). However, little information regarding the transport mechanism of chelated lead species have been yielded by such investigations.

1.5. MODELS THAT DESCRIBE THE TRANSPORT OF LEAD ACROSS THE SMALL INTESTINE

Three models of lead transport may be constructed from the reported data, each will be discussed briefly. The first is an active transport mechanism that involves a high molecular weight mucosal binding protein. The protein is believed to translocate lead across the epithelial cell membrane (i.e. a TRANSCELLULAR ROUTE), into the cell interior. Barton et al., (1978) and Conrad et al., (1978) have suggested that the protein involved is a calcium-binding protein (CaBP) and that in the presence of low calcium concentrations, lead utilises the transport mechanism, and thus there is an antagonism between calcium and lead transport. Other authors have suggested that the protein is an iron-binding protein (FeBP), and again there would be competition between lead and iron for binding sites on the protein, (Six and Goyer, 1972).

An alternative model to describe lead transport by the small intestine is postulated by Coleman (1979) and Blair et al., (1979). There are two aspects to the model,

1. a large rapid uptake of lead onto the tissue surface occurs which renders a large proportion of the lead present in the gut lumen immobile. This process, it was suggested, has no further role in the lead transport process, and was thought to be a protective mechanism against a large lead insult.

2. The lead remaining in the intestinal lumen is then potentially available to diffuse passively across the intestinal epithelium possibly by a paracellular route. The work of other authors may be explained by this mechanism, especially that of Meredith et al., (1977) who propose that a common pathway exists for the absorption of calcium, lead and other minerals.

The third model, a combination of both passive diffusion and active carrier mediated lead transport, is proposed by Aungst and Fung (1981). It is suggested that the relative contribution of the active and passive components of the absorption mechanism will depend upon the concentration of lead in the gut lumen. The authors report that the active component of transport becomes saturated at approximately 10^{-5}M lead, and at lead concentrations in excess of this, the passive component of lead transport becomes significant. They also suggest that the active aspect of the mechanism may be the same as that for iron or calcium active transport.

1.6. AIMS OF THE PRESENT RESEARCH

It is clear that not all of the reported data on lead transport can be rationalised by any one of the three proposed models. It is therefore the aim of the present research to :-

1. Conduct definitive experimentation to determine the extent

to which lead absorption is dependent upon metabolic energy;

2. to determine whether there is a saturation point for the process of lead absorption, and if so to rationalise such a saturation in terms of a binding protein;

3. to investigate the reported antagonism between lead absorption and the absorption of other ions;

4. to determine whether lead transport is via a PARACELLULAR or TRANSCELLULAR route, or a combination of both;

5. and finally, to develop a model that can incorporate some of the reported observations of lead transport to date, and further predict conditions that will either increase or decrease the rate of lead absorption.

CHAPTER TWO

METHODS AND MATERIALS USED

2.1. PHYSIOLOGICAL BUFFER SOLUTIONS

Krebs and Henseleit (1932) bicarbonate buffer containing the following final concentrations of reagents; sodium chloride (112 mM) potassium chloride (4.5 mM), calcium chloride (2.4 mM), potassium dihydrogen phosphate (1.12 mM), and magnesium sulphate (1.12 mM) was used in this study. The solution was adjusted to pH 7.4 with sodium bicarbonate (2.4 mM). Precipitation of calcium carbonate was prevented by gassing the sodium bicarbonate solution with a mixture of oxygen and carbon dioxide (95% : 5%) before mixing. Glucose was added to make a final concentration of 20 mM. This solution is referred to as K.H.B. buffer.

The buffer referred to as Tris consisted of the following reagents; sodium chloride (112 mM), potassium chloride (4.5 mM), calcium chloride (2.4 mM), magnesium sulphate (1.12 mM), tris (hydroxymethane)-aminomethane (5.9 mM), hydrochloric acid (5.0 mM), and glucose (20 mM). The pH of this buffer was 7.2. Incubations carried out in Tris buffer were gassed with oxygen (100%).

A saline buffer was also used, this consisted of a 0.9% (112 mM) sodium chloride solution to which glucose (20 mM) was added, (pH 7.0). Incubations in saline were gassed with oxygen (100%).

Materials used throughout these studies were Analar grade reagents, and were obtained from either Sigma Company Limited, B.D.H. Limited, Fisons Limited or Ciba-Ceigy.

2.2. ANIMAL CONDITIONS

Male Wistar rats (Bantin and Kingman Limited) that weighed between 180 and 200 g were used during these studies. The animals were fed a diet of Heygates 41 B and water *ad libitum*, and kept in an animal house at 20°C. They were allowed water but not food during the 18 hours before sacrifice.

2.3. PREPARATION AND INCUBATION OF EVERTED SACS

The *in vitro* everted sac technique of Wilson and Wiseman (1954) was used extensively throughout these studies.

The animals were stunned by a single blow to the back of the head, and the neck was immediately broken. The process took 2-3 seconds. The intestine was exposed by a mid-line incision in the abdominal cavity and the small intestine put on one side. A cut was made at the ligature of Trietz thus separating the duodenum from the jejunum, and a second cut made at the ileo-caecal junction, and the whole of the small intestine removed. 8 cm consecutive sections were then excised from the small intestine at a distance between 16 and 40 cm from the proximal end, and immediately immersed in

continuously oxygenated K.H.B. buffer, (< 5°C). No more than three sections of small intestine were used from any one animal.

Each section of intestine was everted over a glass rod (3 mm diameter), care being taken not to over-stretch or mechanically damage the mucosal surface. One end of the section was tied with cotton thread and weighed (W_1) on a torsion balance (R. W. Jennings and Company Limited). Forceps and a blunted hypodermic needle were used to introduce approximately 0.5 ml of previously oxygenated buffer into the sac, the open end was tied and the sac reweighed (W_2). During the filling operation the tissue was rested on buffer-saturated filter paper to prevent drying out of the tissue and possible mechanical damage. The total time of sac preparation was kept to a minimum to prevent undue damage, the time taken to prepare three sacs was approximately 3-4 minutes.

In all experimentation described in this thesis, everted sacs were placed in 10 ml of buffered medium oxygenated with O_2 (95%) CO_2 (5%), and shaken at 80-90 oscillations per minute during incubation at 37°C in a water bath, (Griffin Limited).

After incubation sacs were removed from the flasks, blotted by a standardised procedure (on a moist Whatman's No. 1 filter paper), and weighed (W_3). The sacs were then cut, emptied, and drained into a sample bottle and weighed finally (W_4). The fluid taken up by the tissue, and that transported to the serosal compart-

ment was calculated from the following equations.

$W_3 - W_2$ = total fluid taken up

$W_4 - W_1$ = fluid taken up by the tissue

$(W_3 - W_2) - (W_4 - W_1)$ = fluid transported to the serosal compartment

The mass of the threads was found to be negligible compared to the mass of the tissue (< 0.1%).

2.4. THE MEASUREMENT OF LEAD TRANSPORT

Lead transport was assessed by measurement of the gamma-radiation (279 KeV) emitted from the radioisotope ^{203}Pb, (obtained from the Medical Research Council Cyclotron Unit, Hammersmith Hospital, London). The radiation was detected in a Nuclear Enterprises NE 8312 gamma-counter. The error in measurement due to radioactive decay, (half-life of ^{203}Pb is 52.4 hours), was corrected to the nearest half hour by the following equation.

$$A = A_o e^{\lambda t}$$

Where A is the counting rate at time t,

A_o is the counting rate at time t_o,

and λ is a decay constant

The technique depends on the fact that when an appropriate radioactive tracer is thoroughly mixed with an unlabelled compound, the amount of activity per gram of substance will be reduced. If the reduction in activity per gram can be measured, the amount of diluting material added can be calculated. This principle was applied to the measurement of lead transport by everted sacs in the following way. A negligible mass of carrier free $^{203}Pb\ Cl_2$ was added and thoroughly mixed to buffer solutions which contained various known concentrations ($10^{-7} - 10^{-1}$ M) of lead acetate. The final activity of the solutions was 0.2 μCi per ml. Standard volumes of the radiolabelled buffer solutions were measured for radioactivity and a calibration curve drawn. From the calibration curve a value for the activity per gram of lead was calculated. This value was used to convert the activity of tissue and serosal samples into the amount of lead present.

The recovery of radioactivity

In order to measure the recovery of radioactivity, measurements were taken of the radioactivity associated with incubation media before and after a 30 minute incubation.

Activity associated with the tissue and serosal space	approximately 24%
Activity associated with the blotting paper	approximately 5%

Activity remaining in the mucosal bathing solution after incubation	approximately 60%
Total recovery of activity	89%

The loss of accounted activity is probably due to the interaction of ^{203}Pb with the glassware. Confirmation of this conclusion was obtained when it was found that approximately 10% of the radioactivity was associated with the glassware after buffer solutions containing ^{203}Pb were allowed to stand for 24 hours. Haissinsky, (1964) has suggested that lead probably binds to the silicate groups of the glassware. The data was corrected for this discrepancy.

2.5. ESTIMATION OF METHIONINE BY BETA-SCINTILLATION COUNTING

L-(methyl-^{14}C) methionine (Radiochemical Centre, Amersham) was estimated in 200 µl samples of the serosal fluid diluted to 1 ml with distilled water, and added to 10 ml of scintillant. The scintillant comprised of the following reagents; toluene, 1 litre; emulsifier, 500 ml; 2,5-diphenyloxazole (PPO), 5 g ; and 1,4-bis (5-phenyloxazol-2-yl) benzene (POPOP), 0.1 g.

The samples were counted for 10 minutes or 10,000 counts

in a Beckman LS 7500 beta-counter, corrections for quenching were made automatically.

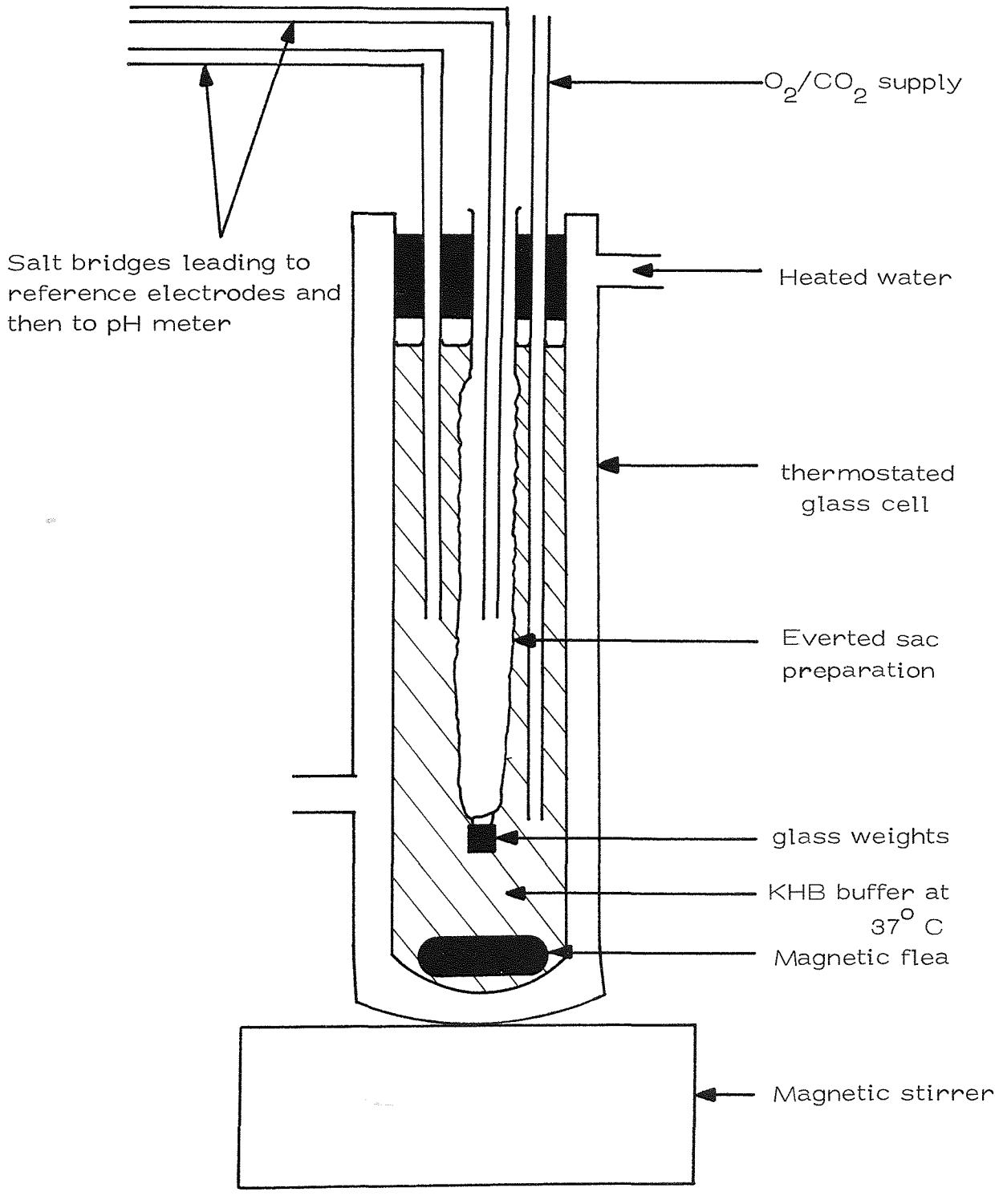
Tissue samples were weighed and dried overnight (12 hours) in a freeze-drying machine (Virtis Techmation Limited). They were reweighed and approximately 100 mg of the dried tissue was put into a porcelain boat, and burnt in a stream of oxygen in a Beckman Biological Material Oxidiser. The machine was allowed to reach its operation temperature before use. ^{14}C-material was converted to $^{14}CO_2$ and collected in 15 ml of Fisosorb '2' (Fisons Limited), and counted for beta-activity in the same counter.

2.6 THE MEASUREMENT OF TRANSMURAL POTENTIAL DIFFERENCE

Potential difference was measured by a method based on that of Barry et al., (1964). A section of small intestine was everted as previously described and tied onto the end of a glass cannula. The lower end of the section was tied and allowed to hang vertically with small glass weights. The sac was filled using a syringe with K.H.B. buffer, care was taken to ensure that no air was trapped in the sac or cannula. The cannulated sac was then completely emmersed in 10 ml of continuously oxygenated buffer contained within a specially designed glass cell, (Figure 2.1).

To obtain a potential difference reading a salt bridge was

Figure 2.1 The cell used for the measurement of transmural potential difference



placed inside the glass cannula, so that it rested approximately half way down the sac. A second salt bridge was placed into the mucosal bathing medium so that it was at the same level. The other ends of the salt bridges were placed in separate solutions of saturated KCl with calomel reference electrodes. The two electrodes were connected to a digital voltmeter so that a circuit was made across the wall of the cannulated sac. During incubation the salt bridges were not disturbed, and readings taken at five minute intervals for a period of one hour.

Salt bridges were made by filling polyethylene tubes (40 cm by 2 mm), with Agar-Agar (5%) dissolved in potassium chloride (3M) at 60^{o}C, and allowing the tubes to cool to room temperature. The salt bridges were tested as a bridge between two calomel electrodes in conjunction with a digital voltmeter (PW 2409, Pye-Unicam, Cambridge). Before potential difference measurements were taken, the voltmeter was calibrated so that the reading across any salt bridge was zero $\pm$ 0.5 mV.

2.7. THE ESTIMATION OF GLUCOSE

Mucosal and serosal fluid samples (200 µl volume) were analysed to determine the amount of glucose transported during various periods of incubation.

A colorimetric glucose oxidase method was used which

involved the use of a bound enzyme as part of a Technicon Autoanalyser system, (Ames Company, Slough). The principle of the method is:-

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \delta\text{-Gluconolactone} + H_2O_2$$

$2\,H_2O_2$ + 3,5-dichloro-4-hydroxybenzene

sulphate + 4-aminophenazone

↓

Quinoneimine (purple colour, extinction at 505 nm)

The technicon Autoanalyser was calibrated with a series of glucose solutions of known concentration, and the resultant peak heights compared with those obtained from the sample solutions.

2.8. HISTOLOGICAL PREPARATIONS

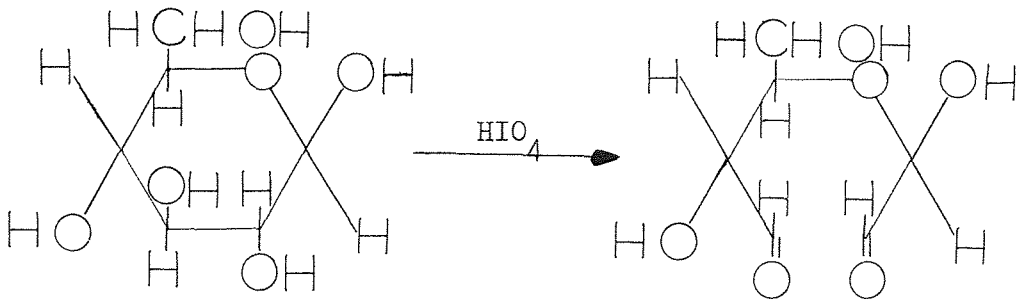
The histology presented was carried out in conjunction with staff of the Department of Histology, The General Hospital, Steelhouse Lane, Birmingham.

A small portion of an everted sac was put into 2 ml of saline (0.9%) which contained 10% formaline as a fixative for 12 hours. The fixed material was progressively dehydrated by immersion in alcoholic solutions (50%, 70% and 100% alcohol respectively), over a period of 24 hours. A process of repeated washings in chloroform for two hours followed. The material was then embedded in a paraffin wax (melting point 57°C) for a minimum of four hours.

A sledge microtome (type 1400, Leitz, West Germany) was used to cut sections (400 nm thick) which were mounted on microscope slides. The sections were washed in xylene to dissolve away the paraffin wax, and stained. Four types of stain were used for the work presented in this thesis, Periodic Acid Schiff (P.A.S.), Haemotoxin and Eosin, Chromate, and Rhodizonate.

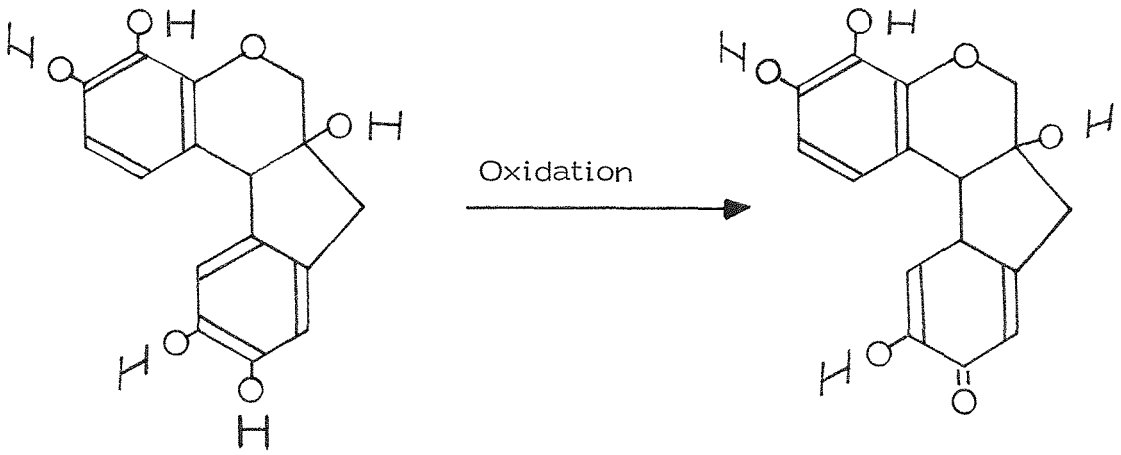
Periodic Acid Schiff

The Periodic Acid Schiff (P.A.S) reaction is used to stain carbohydrate, polysaccharides and muco substances. The theoretical basis of the P.A.S. reaction is essentially that adjacent 1:2 glycol groups (CHOH-CHOH) are converted by periodic acid into two aldehyde groups (CHO); these are demonstrated with Schiff's reagent.



Haematoxylin

Haematoxylin is used in conjunction with eosin to stain nuclei, cartilage, basophilic cytoplasm, muscle fibres and collagen. It is a natural dye which on interaction with various tissue components oxidises and gives rise to a characteristic colour.



(Characteristic Colour)

Chromate and Rhodizonate method for lead salts

The basis of these methods is that lead will displace the sodium from the chromate and rhodizonate salts to form the corres-

ponding insoluble lead chromate (yellow crystals) or lead rhodizonate (scarlet red).

2.9. PREPARATION OF MUCOSAL SCRAPES

Jejunal sacs prepared and incubated in lead acetate (and ^{203}Pb), as previously described, were drained, laid flat on a moist filter paper and the edge of a glass microscope slide drawn longitudinally along their upper surface. The sac was turned over and the scrape procedure repeated. The remains of the sac (mainly muscular tissue), together with the scraped material and filter paper were examined both histologically and for radioactivity. The procedure was similar to that described by Dickens and Weil-Malherbe (1941).

2.10. THE SPECTROSCOPIC DETERMINATION of 2,4,6-TRIAMINOPYRIMIDINE (T.A.P)

A full spectrum scan was taken of T.A.P., (to give a maximum optical density of 2.0), in a Unicam SP 1700 Ultraviolet Spectrophotometer, (Pye-Unicam Limited, Cambridge). A single peak was obtained due to an absorption at 270 nm, (in the ultraviolet region). A series of T.A.P. concentrations were prepared and their relative optical densities measured at 270 nm. A calibration curve was plotted from the values obtained, and a value for the Molar Absorptivity was used in conjunction with Beer's law to determine the

concentration of T.A.P. present in samples.

Beer's Law

$$A = a\,b\,c$$

Where A = observed absorbance (optical density),

a = Molar Absorptivity

b = pathlength of the spectrophotometer cell (cm)

c = concentration of sample (moles per litre).

Since $A = a\,b\,c$ and $b = 1$ cm, then

$a = \frac{A}{C}$ which is the gradient of the calibration curve.

Thus since 'a' can be calculated, and 'b' = 1 then the concentration of a sample 'c' can be determined.

2.11 STATISTICAL CALCULATIONS

The results presented in this thesis unless otherwise stated are the mean of six experimental observations with the standard error of the mean. The Student' 't' - test was used to determine whether any differences between a control value and that of a test were significant; i.e. beyond the likelihood of chance. The following formulae were used in arriving at a value for t;

$$\text{Variance} \quad S_1^2 = \frac{N_1 \sum X^2 - (\sum X)^2}{N_1 \ (N_1 - 1)}$$

Estimate for population standard deviation;

$$\sigma = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}$$

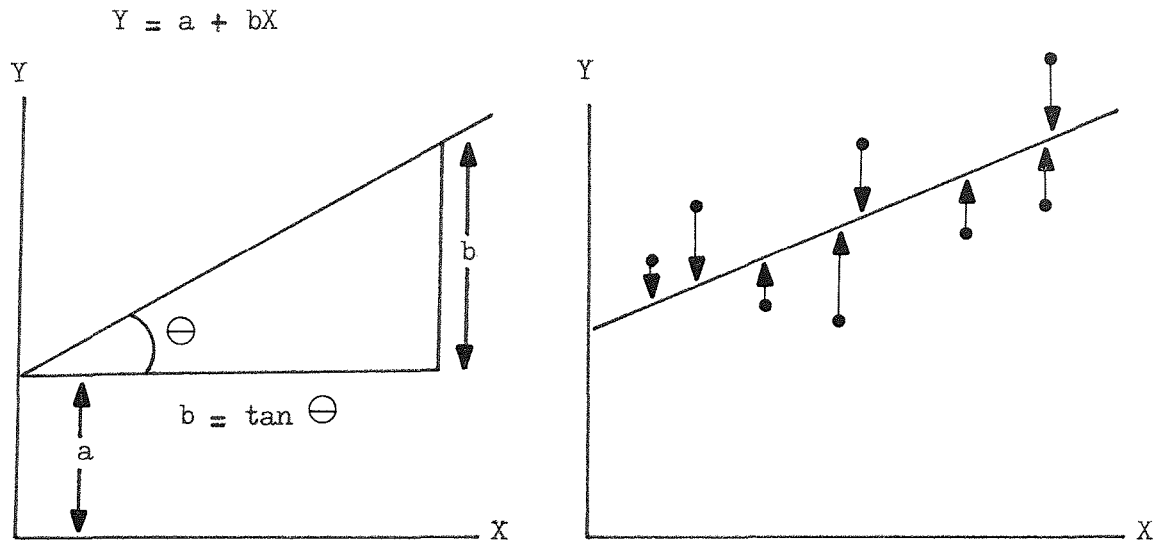
A value for t is obtained from the following expression thus;

$$t = \frac{M_1 - M_2}{\sigma \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Using the value obtained for t the probability of significance was obtained from 'Student's t - tables.

The second statistical technique used was the 'method of least squares' to obtain the best straight line fit from a series of data. The principle is as follows : given a scatter of points and a straight line drawn through them, then the departure of each point from the line (indicated by the arrows) is called a residual. The method of least squares computes the line such that the sum of the squares of the residuals is a minimum.

The following formulae were used in arriving at a value for the correlation coefficient;



Using the following notation,

$\sum x = X, \sum y = Y, \sum x^2 = Q, \sum y^2 = S,$ and $\sum xy = W,$ with N as the number of pairs ;

then the slope $= \dfrac{W - \dfrac{XY}{N}}{Q - \dfrac{X^2}{N}}$

and the intercept $\dfrac{XW - YQ}{X^2 - NQ}$ or by substitution $= \dfrac{Y - X\,(\text{slope})}{N}$

$$\text{The correlation coefficient} = \frac{W - \frac{XY}{N}}{\sqrt{\left(\frac{Y^2}{N} - S\right)\left(Q - \frac{X^2}{N}\right)}}$$

The level of significance was then determined by looking up the value of r from correlation tables.

The above statistical calculations were performed on an Olivetti Programma 101 computer.

Throughout this thesis the following convention was adopted;

Significance of critical probablility levels

Probability (P) of difference being due to chance	Interpretation
P = more than 5%	Significant difference is not proven
P = 5% or less	Difference is significant
P = 0.1% or less	Difference is very significant

CHAPTER THREE

AN ASSESSMENT OF EVERTED SAC VIABILITY FOR ITS USE IN TRANSPORT STUDIES

3.1. METHODS OF ASSESSING VIABILITY

Many difficulties are encountered when investigating natural phenomena. Physiological systems, in particular, are prone to variation due to their inherent complexity and indeed, the very fact that certain systems are being observed takes them from their natural state, and to some extent induces artefacts that, if not carefully interpreted, may give rise to incorrect conclusions.

The ideal investigations would be those where a single parameter at a time could be altered, and the effect observed. However, all techniques of scientific investigation whether *in vivo* or *in vitro* are to some extent a compromise of this basic ideal. For example, the technique of vascular and luminal perfusion for the investigation of transport processes of the intestine may well be envisaged to be a better approximation of the natural state than an *in vitro* technique such as the everted sac. However, when one observes that for the former technique an anaesthetic is used, the effect of anaesthesia on cell metabolism should also be considered since it may retard the very process under investigation. An example of this is the effect of local anaesthetics on the potential difference of the small intestine of the rat, (Dinda and Beck, 1969). Other considerations such as the effect of the nervous system on muscle tone and of hormonal alterations of cell permeability, must be taken into account in the overall interpretation of results and subsequent conclusions.

The everted sac affords certain conditions which allow rigid control of the environment. Since it is an *in vitro* technique there are no great problems arising from nervous or extraneous hormonal control. The absence of a blood vascular system for removing absorbed substances may, if allowed sufficient time, lead to the accumulation of a solute, and thus create an adverse concentration gradient. This would not occur with an *in vivo* technique. This problem can to some extent be overcome by using short incubation times or ensuring the concentration gradients between the mucosal and serosal sides of the sac are maintained.

All techniques have inherent disadvantages and often a particular technique is chosen for its convenience, nevertheless, whatever technique is adopted, the criteria of viability are fundamental and an assessment must be made if relevant conclusions are to be drawn from experimental observations. Those criteria indicative of normal physiological function must, when measured under experimental conditions, approximate to those of the normal state.

The small intestine is essentially an organ of absorption and much study has been devoted to investigating the mechanisms involved. For the purpose of this thesis, certain well-defined absorption processes have been chosen to be representative of normal gut absorptive physiology. Thus by measuring these parameters in the everted sac preparations, an assessment was made regarding

the 'fitness' of the technique for absorption studies.

3.2. THE TRANSMURAL POTENTIAL DIFFERENCE

A readily measured feature of a rat intestinal preparation is its steady transmural potential difference. Many workers have observed the phenomenon of small but significant transmural potentials, ranging from approximately 4 mV (Finkelstein and Schachter, 1962) to 12 mV (Barry et al., 1964). Other species reported demonstrating similar potential differences include man (Gustke et al., 1967), rabbit (Schultz and Zalusky, 1964), dog (Phillips and Code, 1966), bullfrog (Levin, 1967) and goldfish (Smith, 1964). The potential difference is due to charge accumulation that occurs when ion pumps actively transfer ions against their electro-chemical gradients. Other contributions to transmural potentials arise from a charge separation created by selective permeability of membranes, (diffusion potentials); and by the effect of sweeping out ions that have accumulated within membranes, (streaming potentials).

Measurement of transmural potential difference

Everted sacs from five regions of the small intestine were prepared as described in Section 2.3. One end of a sac was tied to a glass cannula, and the other end tied and small glass weights attached. The cannulated sacs were filled with K.H.B. buffer, and emersed in oxygenated K.H.B. buffer (37^{o}C). Salt bridges

were attached and the transmural potential difference measured at five minute intervals for one hour. Full details are given in Section 2.6.

In another experiment tissue was initially incubated at 37°C in glucose-free K.H.B. buffer. After 15 minutes incubation, glucose was added to the mucosal bathing medium so that the overall concentration was 20 mM.

The results demonstrate that over 60 minutes of incubation the intestinal tissue was able to maintain a steady transmural potential difference (Table 3.1. and Figure 3.1). There was no statistical difference between the initial and the final value of the potential difference, (Figure 3.1). Table 3.2 is taken directly from the literature, (Barry et al., 1964), it demonstrates the potential differences obtained in this investigation to be very similar to those obtained by other workers under both in vitro and in vivo conditions.

When glucose was added to a glucose-free medium, the potential difference increased from a mean value of 2.06 ± 0.15 mV to 7.52 ± 0.39 mV, and remained steady. Thus the potential difference was largely restored by the addition of 20 mM glucose. This demonstrated that the transmural potential difference obtained was linked to a metabolic process which required the presence of glucose, (Figure 3.1).

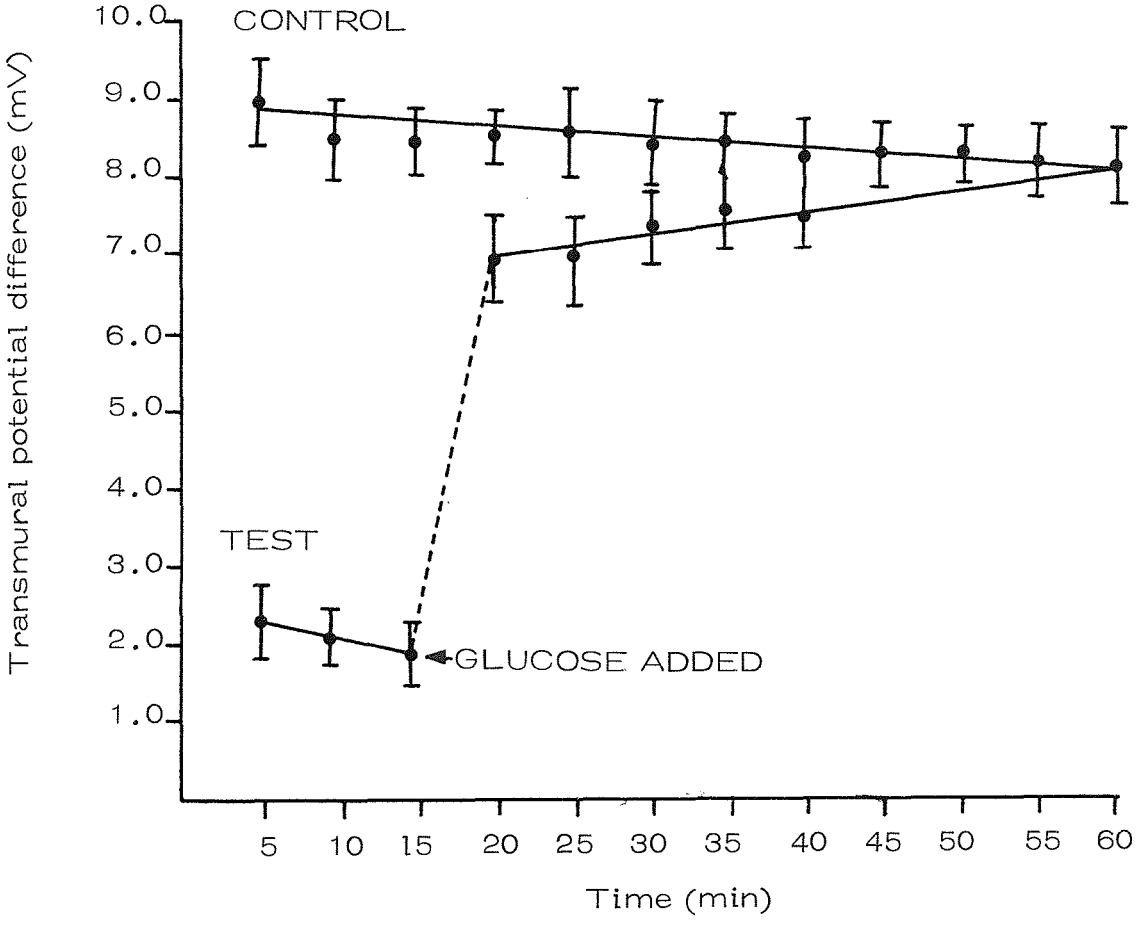
Table 3.1.

The mean potential difference across the epithelium of five regions of the small intestine

REGION OF INTESTINE	MEAN POTENTIAL DIFFERENCE ± S.E. (m V)
Distal duodenum	4.4 ± 0.5
Proximal jejunum	5.0 ± 0.5
Mid-jejunum	8.5 ± 0.4
Proximal ileum	7.1 ± 0.3
Distal ileum	5.3 ± 0.4

Each value is the mean of six experimental observations ± standard error of the mean

Figure 3.1. The transmural potential difference across the mid-jejunum of the rat small intestine in the presence and absence of glucose



Potential difference was measured as described in Section 2.7. Each point is the mean value of six experimental observations ± standard error of the mean

Table 3.2.

LITERATURE QUOTED VALUES FOR COMPARISON PURPOSES WITH EMPIRICALLY DERIVED VALUES FROM THE PRESENT STUDY (From Barry et al., 1964)

	POTENTIAL DIFFERENCE ± S.D. (m V)		
In vitro	Upper jejunum	mid-intestine	lower ileum
No glucose	0.8 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
Glucose (28 mM)	5.8 ± 0.3	12.0 ± 0.2	5.8 ± 0.5
In vivo			
No glucose	2.8 ± 0.4	4.7 ± 0.2	3.8 ± 0.3
Glucose	7.3 ± 0.4	11.1 ± 0.3	7.4 ± 0.7

These data demonstrate that the intestinal preparations can maintain a steady transmural potential difference, and can respond to external metabolic energy, and therefore appear to be physiologically viable.

3.3. GLUCOSE UPTAKE AND TRANSPORT

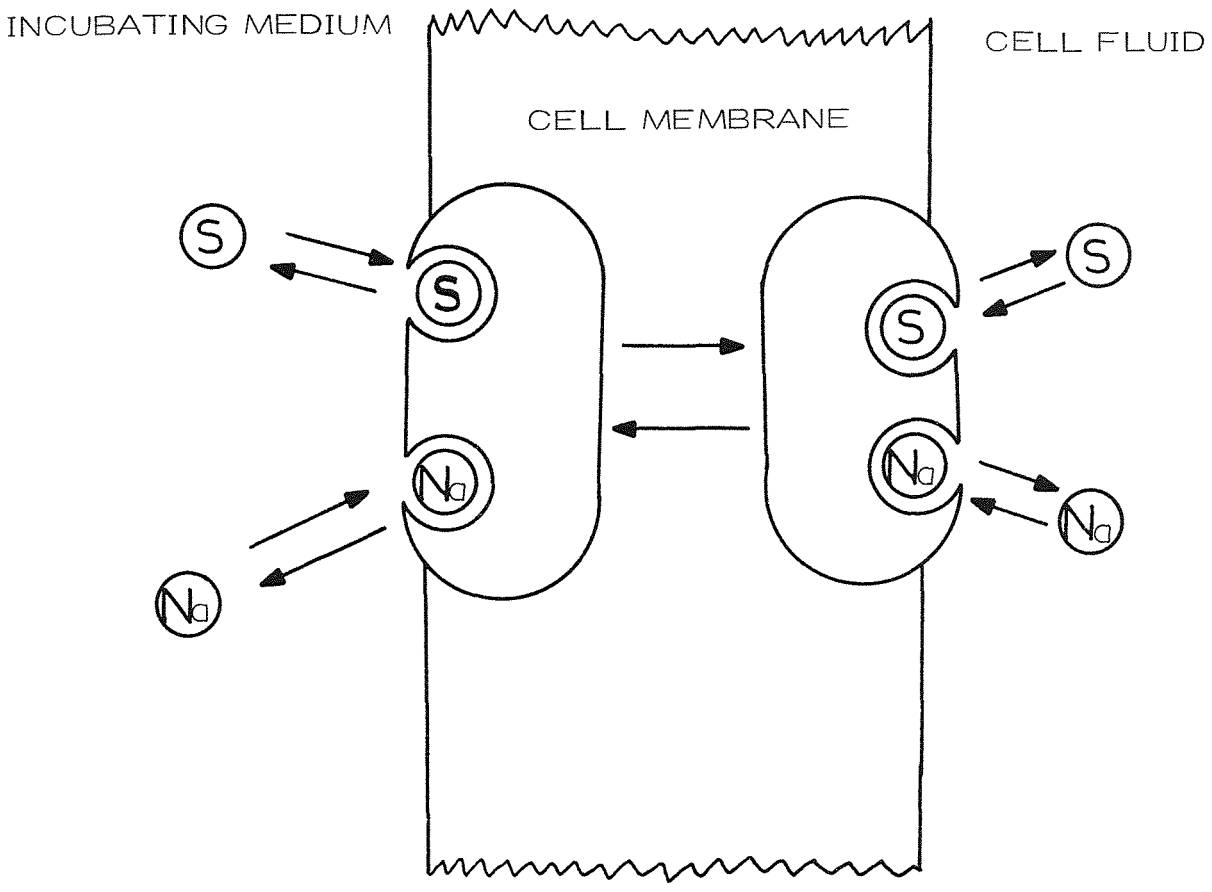
The uptake of glucose by intestinal epithelial cells is believed to occur via a substrate-specific, sodium-dependent and energy-independent mechanism, (Crane, 1962, and Gardner, 1977). The mechanism is postulated (Figure 3.2) to involve a sugar specific carrier located in the intestinal mucosa. The carrier has a second site for binding sodium, and thus movement of glucose into the epithelial cells against its concentration gradient, is facilitated by the movement of sodium into epithelial cells down its concentration gradient. Thus both glucose and sodium are required for the translocation of either species.

Measurement of glucose transport

Everted sacs from five regions of the rat small intestine were incubated at 37^{o}C for 60 minutes in K.H.B. buffer and gassed with O_2 (95%) CO_2 (5%). The glucose concentration of the mucosal bathing medium and the serosal compartment were measured as described in Section 2.7.

The data demonstrated that for four of the five regions of the

Figure 3.2. The postulated mobile carrier and its interaction with substrate and sodium (Crane, 1962)



The mobile carrier has two specific binding sites, one for the substrate (glucose) and the other for sodium. The presence of one is required by the other before translocation can occur. Sodium moves into the cell down its concentration gradient, glucose moves uphill against a concentration gradient.

small intestine investigated, active accumulation of glucose had occurred (a ratio of serosal to mucosal glucose concentration greater than unity indicated active accumulation). The distal ileum was the exception. The greatest accumulation occurred in the mid-jejunum with a S/M ratio of 2.2, (Table 3.3). These data support the previous conclusions that the intestinal preparations are physiologically viable.

3.4. THE INTESTINAL ABSORPTION OF WATER

Diamond and Bossert (1967) proposed a model to explain water transport by the intestine, (the "standing gradient" model). The essential features of the model are as follows. Sodium moves into the mucosal epithelial cell down a concentration gradient. Intracellular sodium is actively pumped across the basolateral membrane into the lateral intercellular spaces where it accumulates and creates an osmotic gradient. Water is induced to enter the lateral intercellular space either from across the cell walls or through the tight junctions. The consequential increase of hydrostatic pressure produces fluid flow into the submucosa (Figure 3.3). At steady-state conditions, a gradient is established along the intercellular channels and fluid moves from the lumen into the submucosal region.

Measurement of water transport

Everted sacs from the mid-jejunum only, were incubated at 37°C for various times (10-60 minutes) in K.H.B. buffer, and gassed with O_2 (95%) CO_2 (5%). Water uptake by intestinal tissue, and water transported to the serosal compartment was measured as described in Section 2.3.

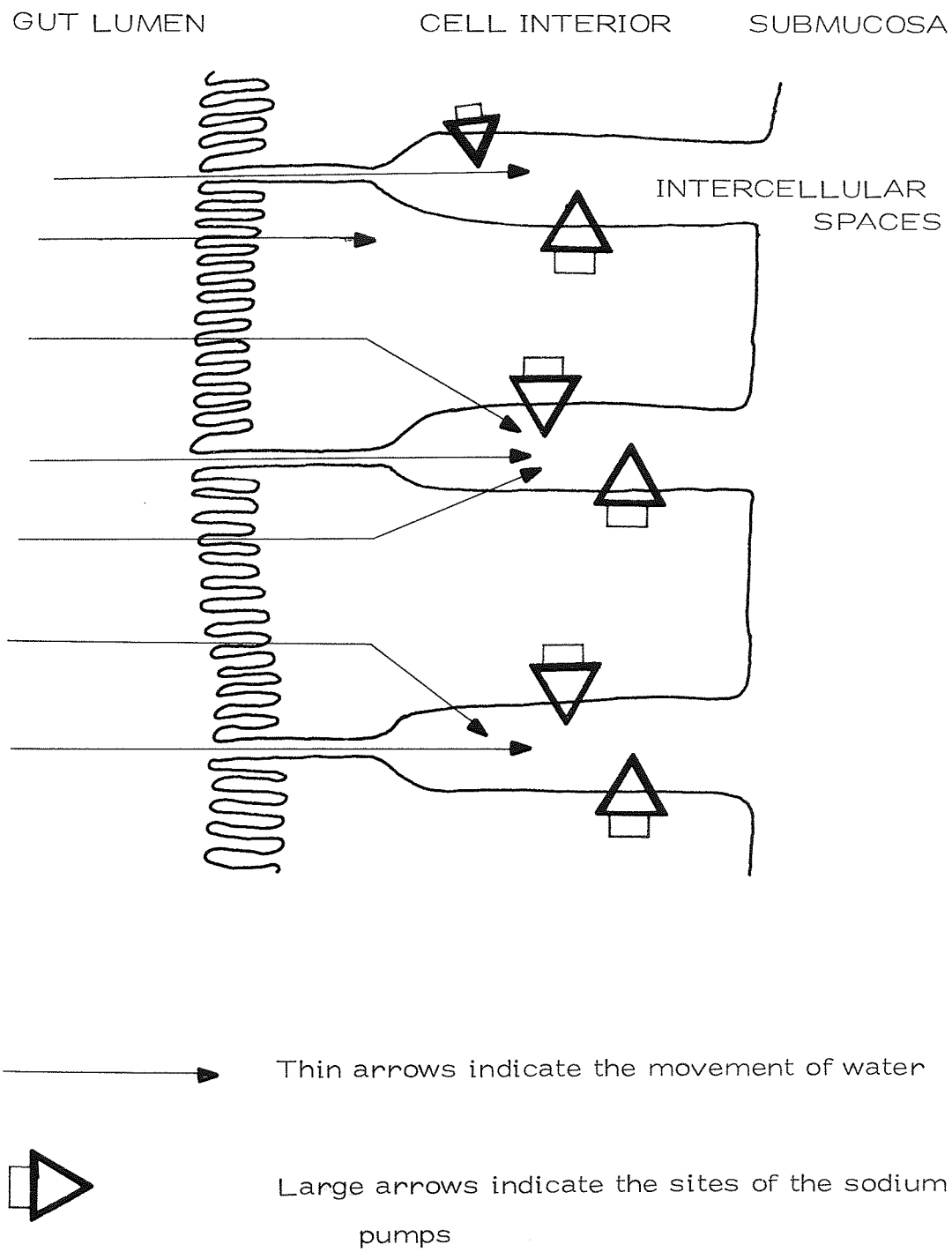
After a short lag of approximately seven minutes the rate of water transport to the serosal compartment was linear ($r = 0.99$, $p < 0.001$) with an overall rate of 1020 $\pm$ 180 µl per gram wet weight of tissue per hour. A linear rate of fluid movement to the serosal compartment is indicative of active electrolyte movement brought about by sodium extrusion from epithelial cells. This process suggests that the intestinal preparations are functioning normally and are therefore viable. The uptake of water by intestinal tissue was rapid for the initial 10 minutes of incubation then more slowly and linear for the duration of the incubation, ($r = 0.98$, $p<0.001$). The rapid initial uptake of water by the intestinal tissue was probably due to water that had entered the mucosal extracellular fluid space, (Jackson et al., 1970) and the formation of an unstirred water layer, (Dietschy et al., 1971 and Winne, 1976). The slower portion of the tissue water uptake process is probably due to water that has entered the cells. The rate of the slower process was

TABLE 3.3. THE MEAN S/M RATIOS FOR GLUCOSE TRANSPORT AFTER 60 MINUTES OF INCUBATION

REGION OF INTESTINE	MEAN GLUCOSE S/M RATIO
DISTAL DUODENUM	1.1 ± 0.1
PROXIMAL JEJUNUM	1.7 ± 0.1
MID-JEJUNUM	2.2 ± 0.2
PROXIMAL ILEUM	2.0 ± 0.3
DISTAL ILEUM	0.7 ± 0.1

Each value is the mean of six experimental observations ± standard error of the mean

Figure 3.3. The 'standing gradient' model of fluid absorption postulated by Diamond and Bossert (1967)



approximately 120 ± 20 µl per gram wet weight of tissue per hour. If this were the situation, then approximately 10% of the total water absorption is due to uptake by tissue and approximately 90% due to transport to the serosal compartment. These data are comparable with those of other authors, (Pritchard and Porteous, 1977 and Jackson et al., 1970).

3.5. AMINO ACID UPTAKE

It has been demonstrated that amino acids can traverse the gut wall against a concentration gradient, (Wiseman, 1951), and that their accumulation in intestinal tissue can be blocked by dinitrophenol and other uncouplers of oxidative phosphorylation, (Agar et al., 1954). Further, amino acid transport exhibits saturation kinetics and thus it is accepted that amino acid transport is via carrier systems located in the brush border.

L-methionine transport

Everted sacs were incubated at 37°C for various periods of time (10-60 minutes) in K.H.B. buffer that contained L-methionine (10^{-6}M) radiolabelled with L-(methyl-^{14}C)methionine (0.1 µCi ml^{-1}, Radiochemical Centre, Amersham), and gassed with O_2 (95%) CO_2 (5%). The radioactivity associated with the intestinal tissue and the serosal compartment was measured as described in Section 2.5.

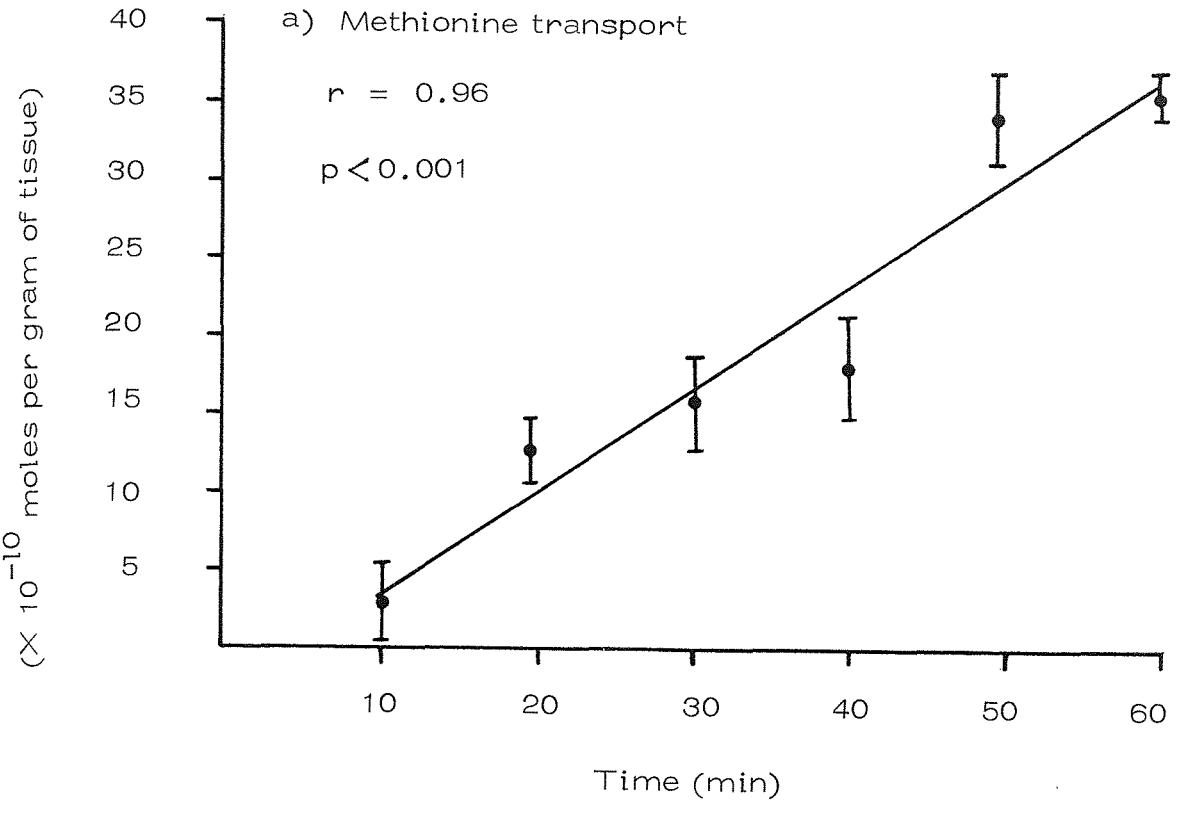
The rates of L-methionine uptake by intestinal tissue and transport to the serosal compartment were linear, ($r = 0.96$, $p < 0.001$, Figure 3.4a, and $r = 0.88$, $p < 0.001$, Figure 3.4b). These data demonstrate typical amino acid uptake characteristics and agree with the data of Robinson and Alvarado (1977). It is concluded that the intestinal preparations are capable of active amino acid absorption and are therefore, physiologically viable.

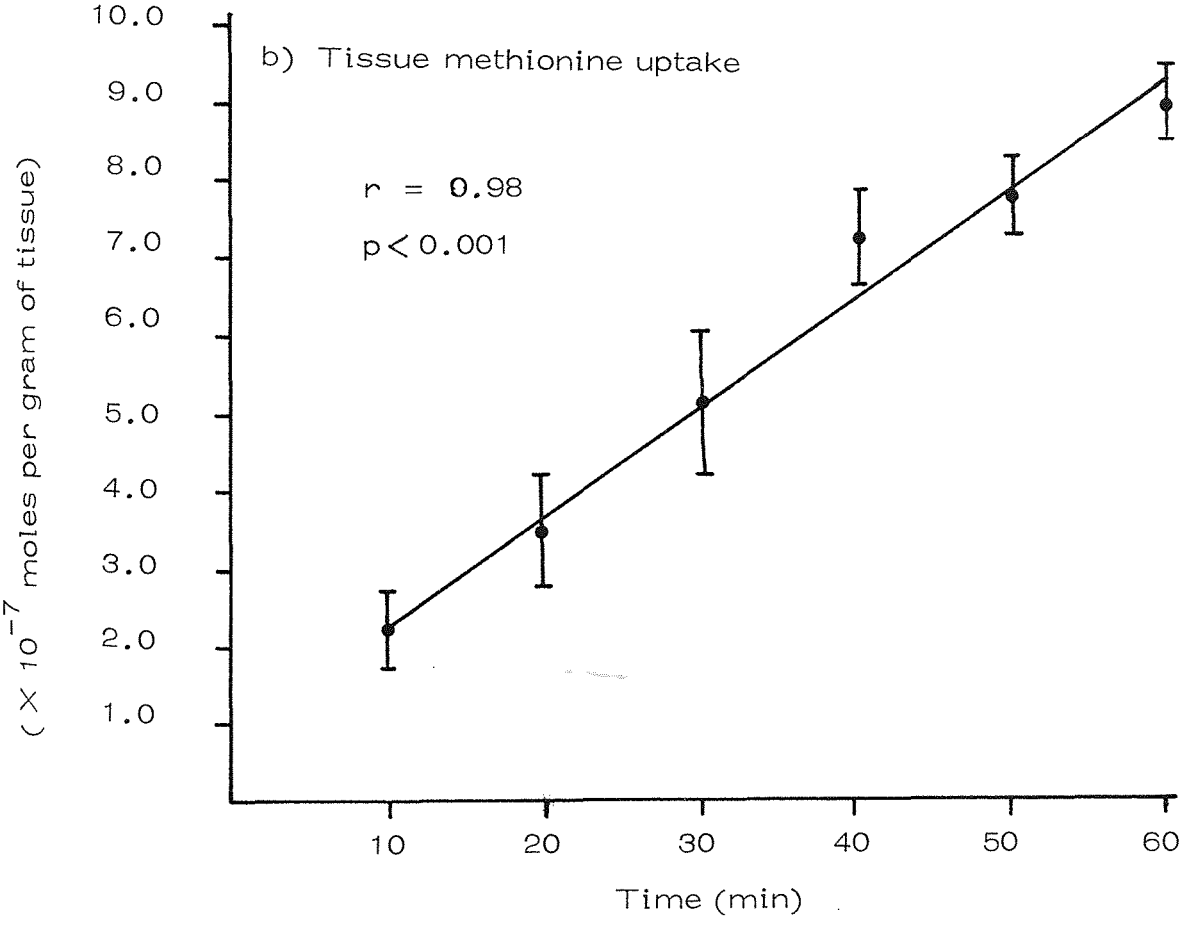
3.6. HISTOLOGICAL ASSESSMENT OF TISSUE INTEGRITY

It has been suggested that the everted sac technique is prone to rapid morphological changes that are apparent after as little as 5 minutes of incubation (Levine et al., 1970). Further, after 30 minutes of incubation 50-75% of the normal epithelium disappear and after 1 hour there is a total disruption of the epithelial border. It has been proposed that the absence of mesenteric lymphatic vessels causes an accumulation of absorbed fluid which subsequently cause cells to swell with consequent major anatomical disruption. Histological examination of incubated everted sacs was conducted to assess the validity of the above suggestions.

Everted sacs were incubated at $37^{o}C$ for 50 minutes in K.H.B. buffer, and gassed with O_2 (95%) CO_2 (5%). Sections were taken and stained with Periodic Acid Schiff (Section 2.8) and photographed through a light microscope. Under low magnification (× 375) there

Figure 3.4. The uptake and transport of L-(methyl-^{14}C) methionine by everted sacs of rat jejunum





Each point is the mean value of six experimental observations ± 1

was no obvious disruption of the villi or loss of epithelial cells, (plate 1). At high magnification (×900) the villi were intact and typical of normal intestinal epithelium, (Plate 2).

The data presented in Section 3.4. demonstrate that the total tissue water uptake after 1 hour of incubation was 520 µl per gram of tissue. Of this approximately 300 µl could be attributed to the mucosal extracellular fluid space (Section 4.5), and a further possible 80 µl to the formation of unstirred layers. Thus a maximum of 140 µl of fluid per gram of tissue, which is 14% of the initial tissue mass, is available to cause cell swelling and disruption. It is unlikely that an increase of tissue volume of 14% would be sufficient to cause a great deal of disruption, and indeed, plates 1 and 2 substantiate this view.

3.7. CONCLUSIONS OF THE VIABILITY ASSESSMENT

The everted sac preparations used throughout this thesis have been demonstrated to be physiologically viable as assessed by the following criteria.

1. The ability to create and maintain a steady transmural potential difference.

2. The ability to actively accumulate glucose.

PLATE 1

Everted sacs were incubated at 37°C in Krebs-Henseleit Bicarbonate buffer for a period of 50 minutes. Sections were taken and stained with P.A.S. reagent as described in Section 2.8.

Magnification approximately x 375

PLATE 2

Everted sacs were incubated at 37°C in Krebs-Henseleit Bicarbonate buffer for 50 minutes. Sections were taken and stained with P.A.S reagent as described in Section 2.8.

Magnification approximately x 900

PLATE 1

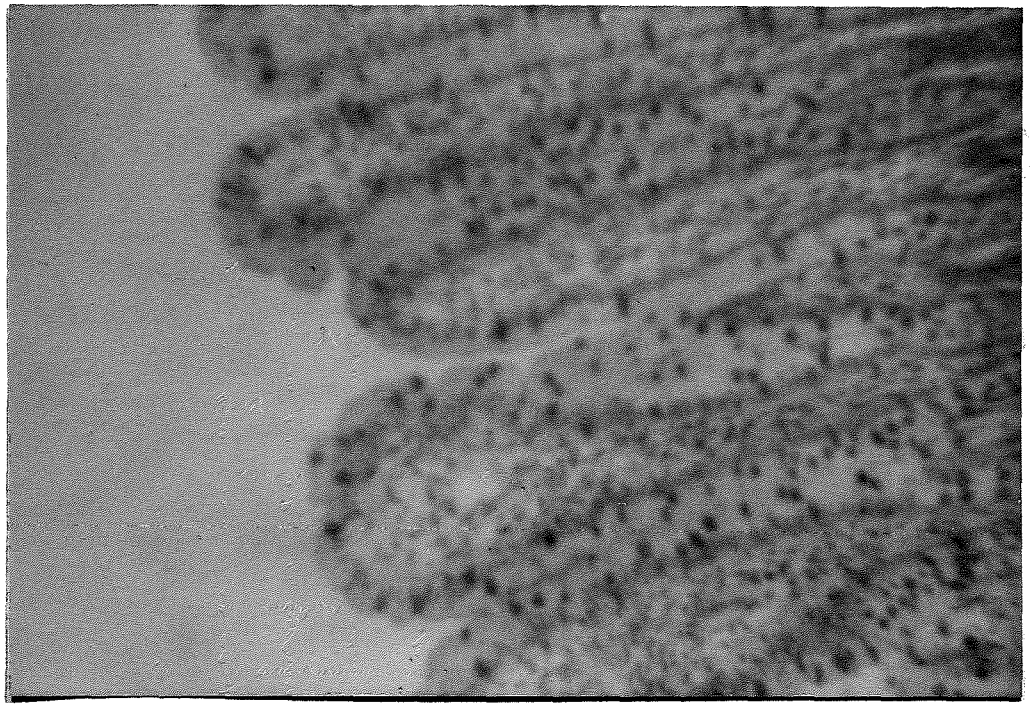
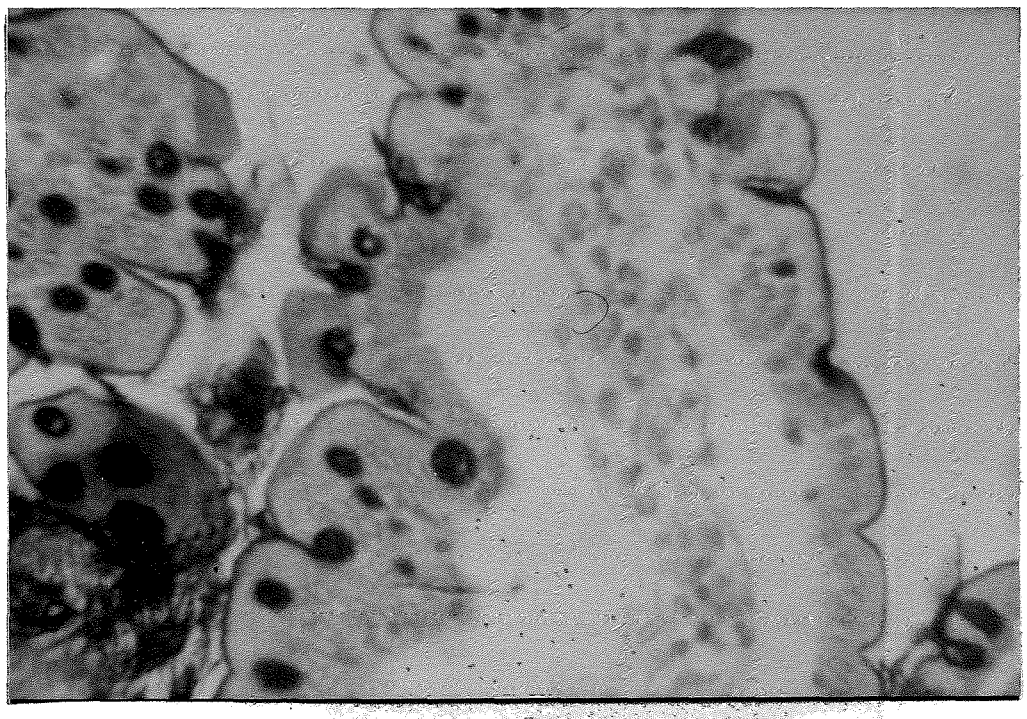


PLATE 2



3. The ability to produce a linear rate of water transport.

4. The ability to take up and transport an amino acid.

5. The preparations were also shown to be histologically intact after incubation.

Many other authors have used everted sac preparations and have demonstrated the technique to be a suitable one for investigation of absorption phenomena. Recent publications of most relevance include Aungst & Fung, 1981, Middleton, 1981; Thomson and O'Brien, 1981, Brantbar et al., 1981 and Pritchard and Porteous, 1977.

CHAPTER FOUR

THE KINETICS OF LEAD ABSORPTION

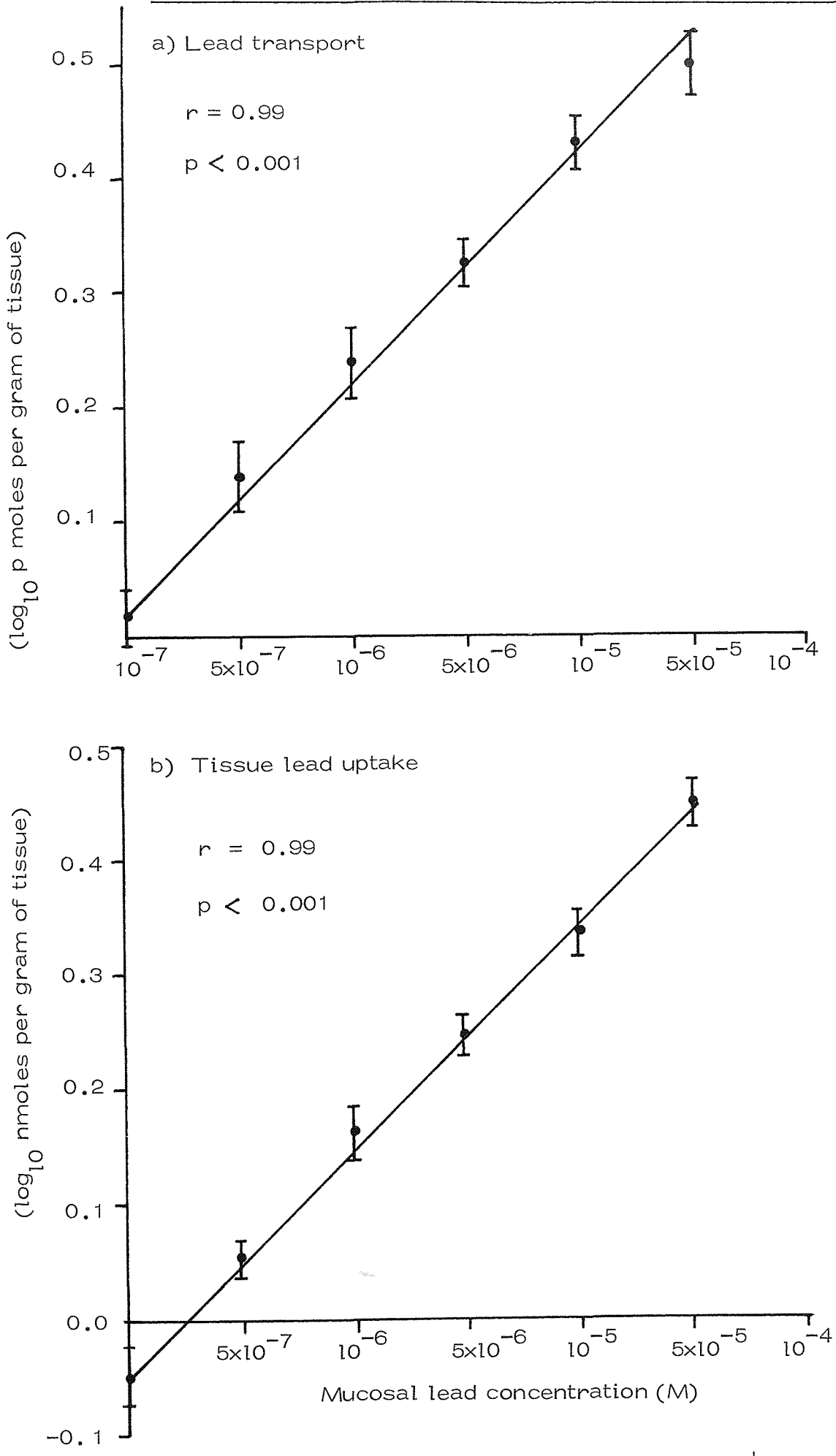
4.1. THE UPTAKE OF LEAD BY INTESTINAL TISSUE AND SUBSEQUENT TRANSPORT

The characteristics of lead absorption by the rat small intestine were investigated over a broad concentration range (10^{-7}M–10^{-1}M). The presence of carbonate and phosphate ions in K.H.B. buffer limit its ability to dissolve lead and causes precipitation, since the maximum solubility of lead carbonate is 4.1×10^{-6}M, and lead orthophosphate is 1.7×10^{-7}M. To overcome this limitation, incubations were conducted in Tris buffer which permitted the use of high concentrations of lead.

Everted sacs were incubated at 37°C for 30 minutes in either K.H.B. or Tris buffer which contained various concentrations of lead acetate (10^{-7}M – 10^{-1}M) and ^{203}Pb as a tracer. K.H.B. buffer solutions were gassed with O_2 (95%) CO_2 (5%), and Tris buffer solutions gassed with O_2 (100%). The lead associated with the intestinal tissue, (hereafter referred to as tissue uptake), and that transported to the serosal compartment, (hereafter referred to as lead transport), was measured as described in Section 2.4.

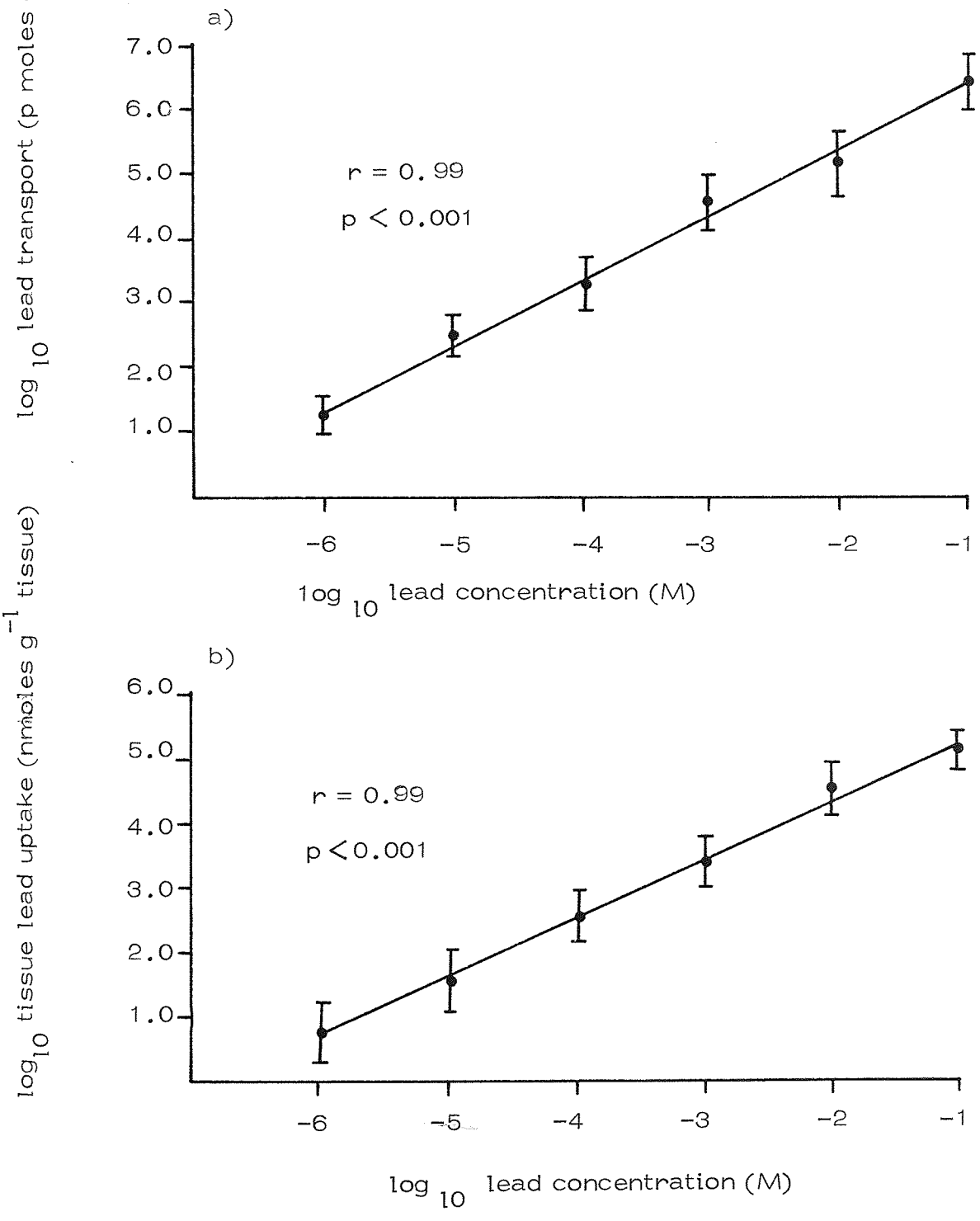
The rate of lead transport and of tissue uptake was dependent upon the initial lead concentration present in the incubating medium, and increased linearly as the lead concentration increased; ($r = 0.99$ Figure 4.1a, $r = 0.99$ Figure 4.2a, $r = 0.98$ Figure 4.1b and $r = 0.99$ Figure 4.2b). There was no indication of saturation of

Figure 4.1. A concentration based investigation of lead uptake and transport in Krebs-Henseleit Bicarbonate (K.H.B) buffer



Each value is the mean of six experimental observations $\pm$

Figure 4.2 A concentration based investigation of transport and tissue uptake of lead in Tris buffer



Each value is the mean of six experimental observations ± standard error of the mean

either process.

Further incubations were conducted at different time periods and at lead concentrations comparable to dietary levels present in the gut of normal man, (approximately 10^{-6} M). Everted sacs were incubated at 37°C for various times (10-60 minutes) in K.H.B. or Tris buffer that contained various concentrations of lead acetate (10^{-7}M - 10^{-5}M) labelled with ^{203}Pb, and gassed with oxygen and carbon dioxide (95%: 5%) or oxygen (100%) as before. The tissue uptake and transport of lead were measured as described Section 2.4.

The rate of lead transport was linear with time, (Figures 4.3 a, 4.3 b, 4.3 c and 4.5 a). Extrapolation of the regression lines to the Y axis produced an intercept above the origin.

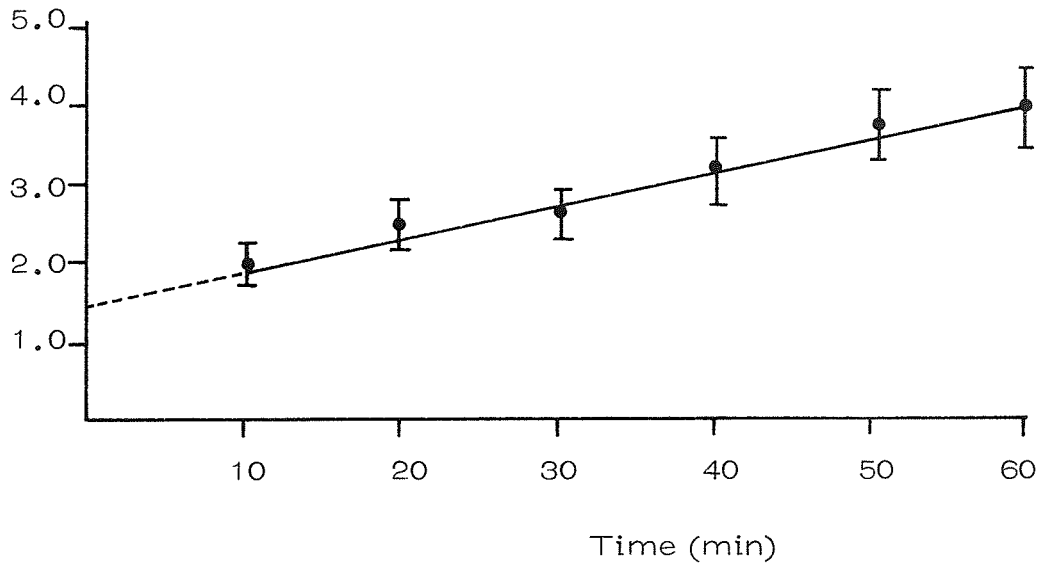
Tissue uptake of lead appeared to be almost complete within the initial ten minutes of incubation, thereafter the tissue accumulated lead very slowly, (Figures 4.4 a, 4.4 b, 4.4 c and 4.5 b). After 60 minutes of incubation approximately 20% of the lead present in the incubating medium was taken up by the intestinal tissue, and approximately 0.2% transported to the serosal compartment.

Coleman (1979) demonstrated that both the rate of tissue uptake and transport of lead are dependent upon the initial lead concentration of the incubation medium, and neither process is saturated over the lead concentration range of 10^{-7} M - 5×10^{-5} M.

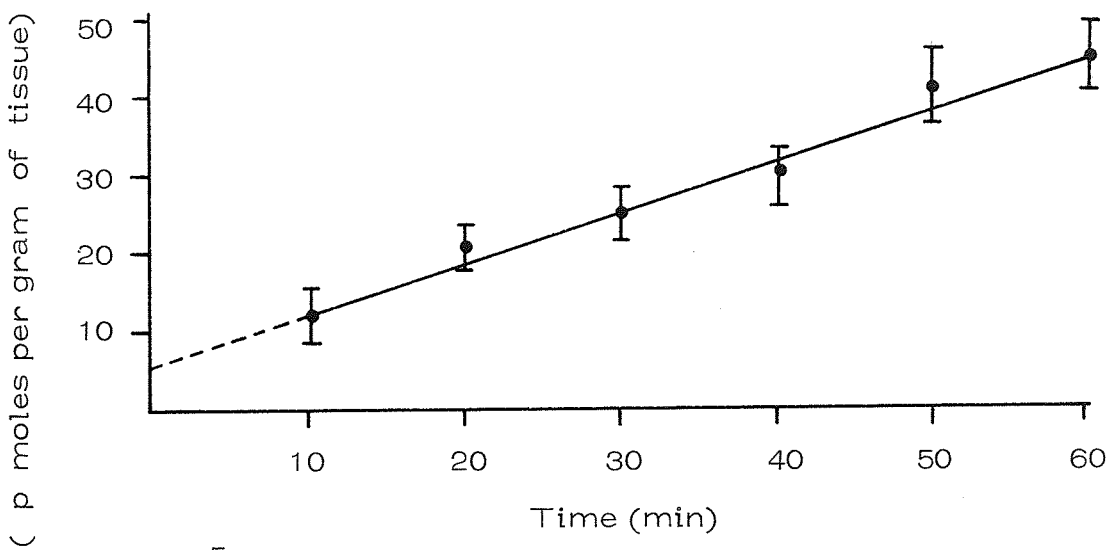
Figure 4.3.

A time based investigation of lead transport in Krebs-Henseleit-Bicarbonate (K.H.B) buffer at three different lead concentrations

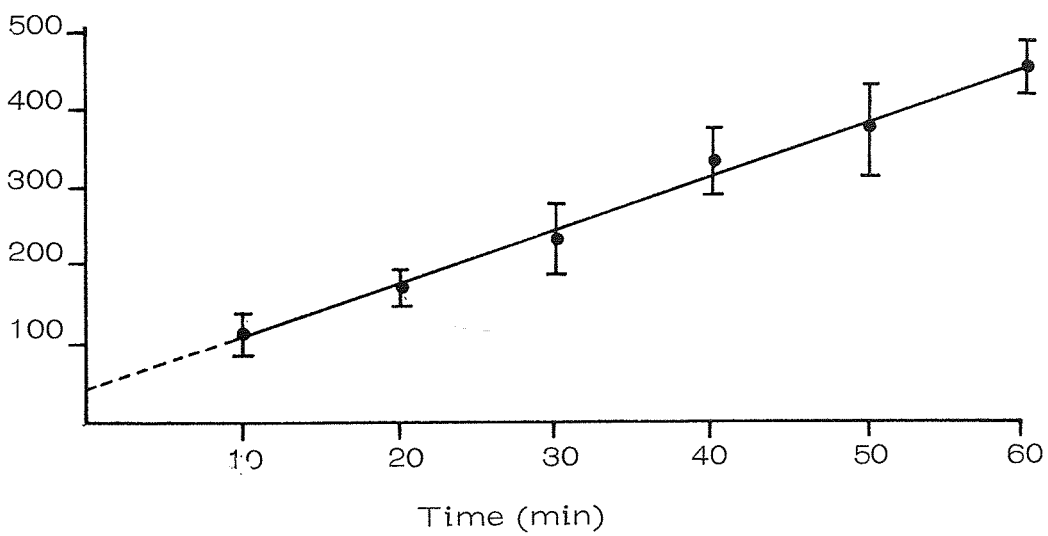
a) 10^{-7} M lead acetate



b) 10^{-6} M lead acetate



c) 10^{-5} M lead acetate



Each value is the mean of six experimental observations ± standard error of the mean

Figure 4.4 A time based investigation of lead uptake in Krebs-Henseleit Bicarbonate (K.H.B.) buffer at three different lead concentrations

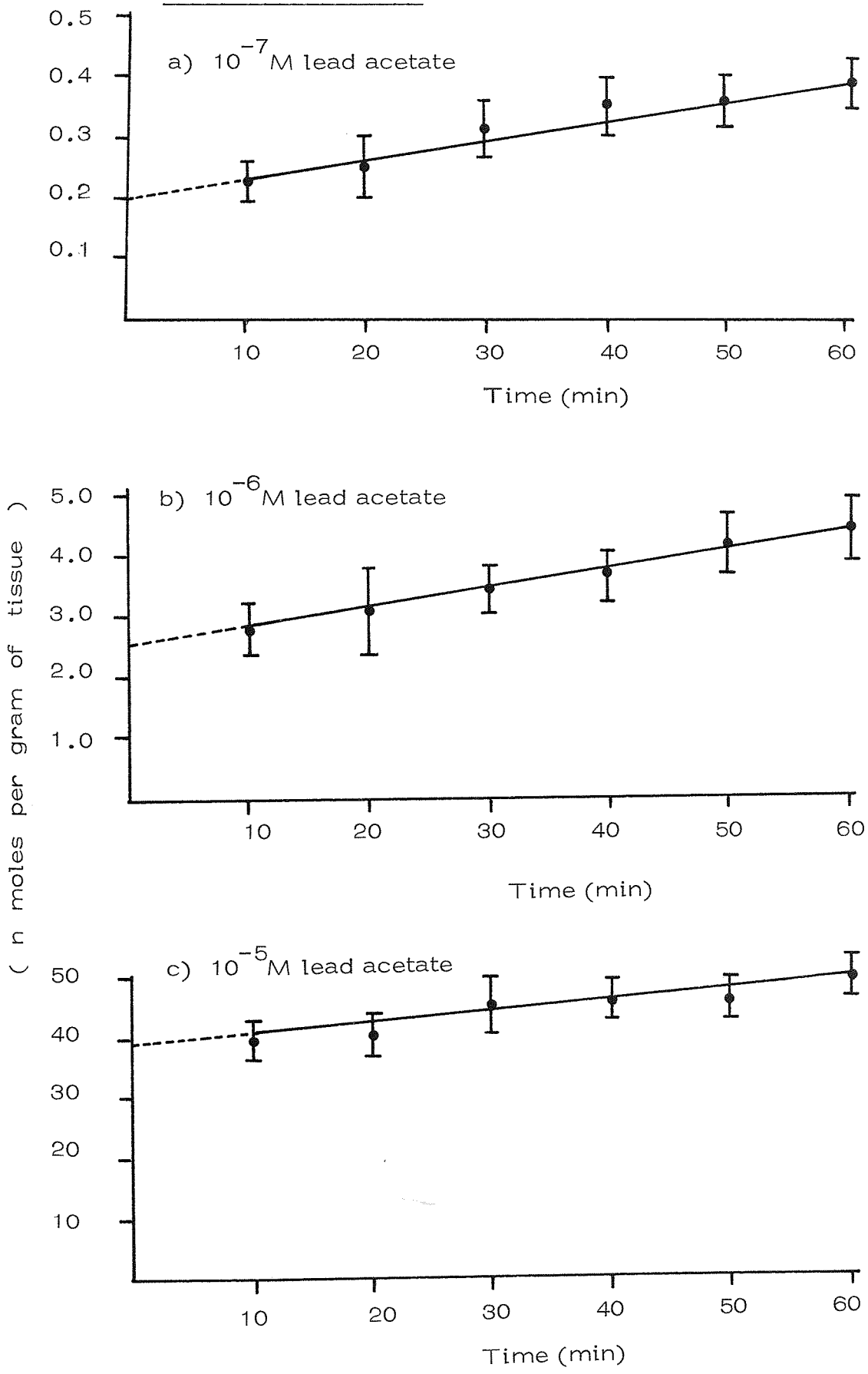
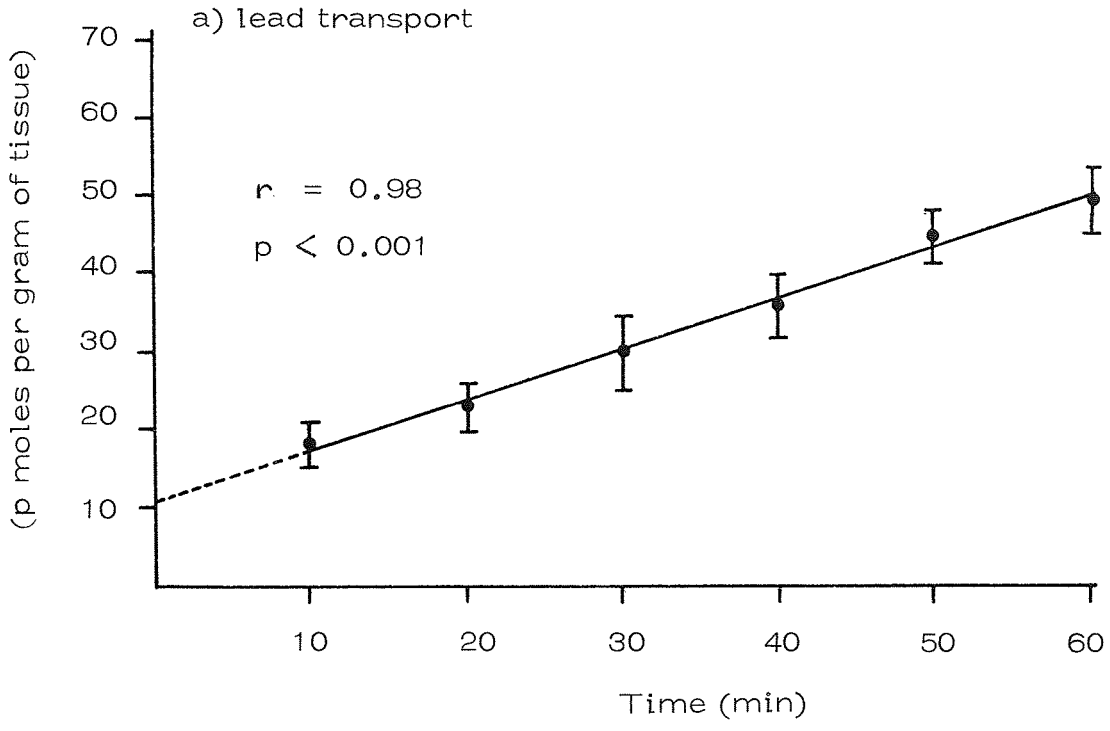
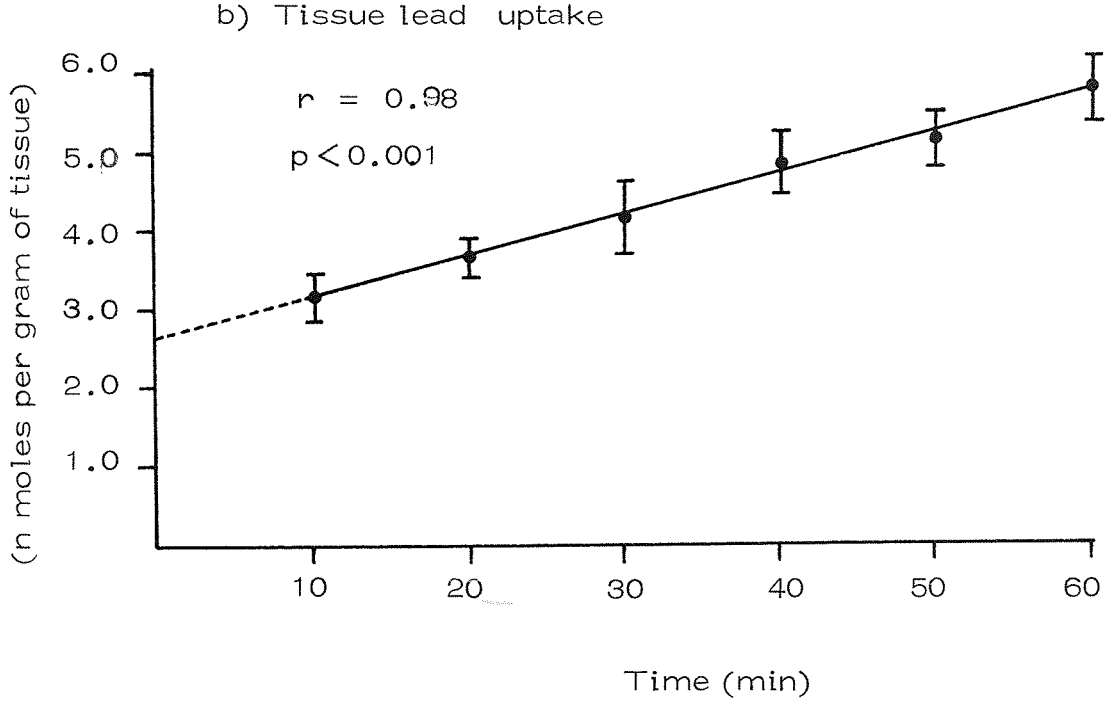


Figure 4.5.

A time based investigation of the transport and tissue uptake of lead in Tris buffer





Each value is the mean of six experimental observations $\pm$ standard error of the mean

Coleman further postulated that lead is rapidly adsorbed onto the intestinal tissue surface, and demonstrated a rapid uptake during the initial 20 minutes of incubation, and no further uptake thereafter. The data of Coleman are confirmed by those reported here, and have been extended to demonstrate non-saturation of the processes of tissue uptake and transport of lead upto mucosal lead concentrations of 10^{-1} M.

4.2. INVESTIGATIONS INTO THE ENERGY REQUIREMENTS OF LEAD UPTAKE AND TRANSPORT

The energy dependence of the lead uptake and transport processes was investigated by incubation either at a lower temperature, or in the presence of metabolic inhibitors, or in the absence of glucose, or under conditions of anoxia.

Everted sacs were incubated at 37^{o}C for 60 minutes in K.H.B. buffer that contained lead acetate (10^{-6}M) and ^{203}Pb, and gassed with O_2 (95%) and CO_2 (5%) under one of the following conditions;

1. in the presence of 2,4-dinitrophenol (10^{-2}M),
2. in the presence of iodoacetate (10^{-2}M),
3. in the absence of glucose, or
4. at a lowered incubation temperature (27^{o}C).

The transport and tissue uptake of lead was measured as described

in Section 2.4.

The absence of glucose or the presence of 2,4-dinitrophenol produced no significant effect on either the tissue uptake or transport of lead, (Table 4.1). The Q_{10} values obtained were 0.8 for lead transport and 1.3 for tissue uptake of lead. The data is in agreement with that presented by Coleman (1979) and leads to the conclusion that the process of tissue uptake and transport of lead are independent of a requirement for metabolic energy. Incubation in the presence of iodoacetate however, caused a significant elevation ($p < 0.02$) of tissue uptake, and a significant decrease ($p < 0.05$) of lead transport. This data may appear to stand in contradiction to those previously presented, but it may imply a role for the acid microclimate (see Section 1.3, Chapter One) which could be a factor in determining the extent of tissue lead uptake, and will be discussed later in this thesis.

4.3. THE EFFECT OF ANOXIC INCUBATION CONDITIONS ON LEAD TRANSPORT

Anoxia is a potent metabolic inhibitor that is known to have a significant effect upon the acid microclimate, (Lucas et al., 1980). For this reason, everted sacs were incubated at 37°C for various times (10-60 minutes) in K.H.B. buffer that contained lead acetate (10^{-6}M) and ^{203}Pb, and gassed with nitrogen and carbon dioxide

TABLE 4.1. THE EFFECT OF VARIOUS PARAMETERS ON THE TRANSPORT AND TISSUE UPTAKE OF LEAD CATIONS

	CONTROL	Q_{10}	NO GLUCOSE	DINITROPHENOL	IODOACETATE	
LEAD TRANSPORT (p moles g^{-1})	53.04 ± 5.57	0.9	50.12 ± 6.66	49.03 ± 5.23	37.43 ± 2.14	$p<0.05$
TISSUE LEAD UPTAKE (n moles g^{-1})	4.78 ± 0.52	1.3	4.60 ± 0.71	5.37 ± 0.35	6.47 ± 0.51	$p<0.02$

Each value is the mean of six experimental observations ± standard error of the mean

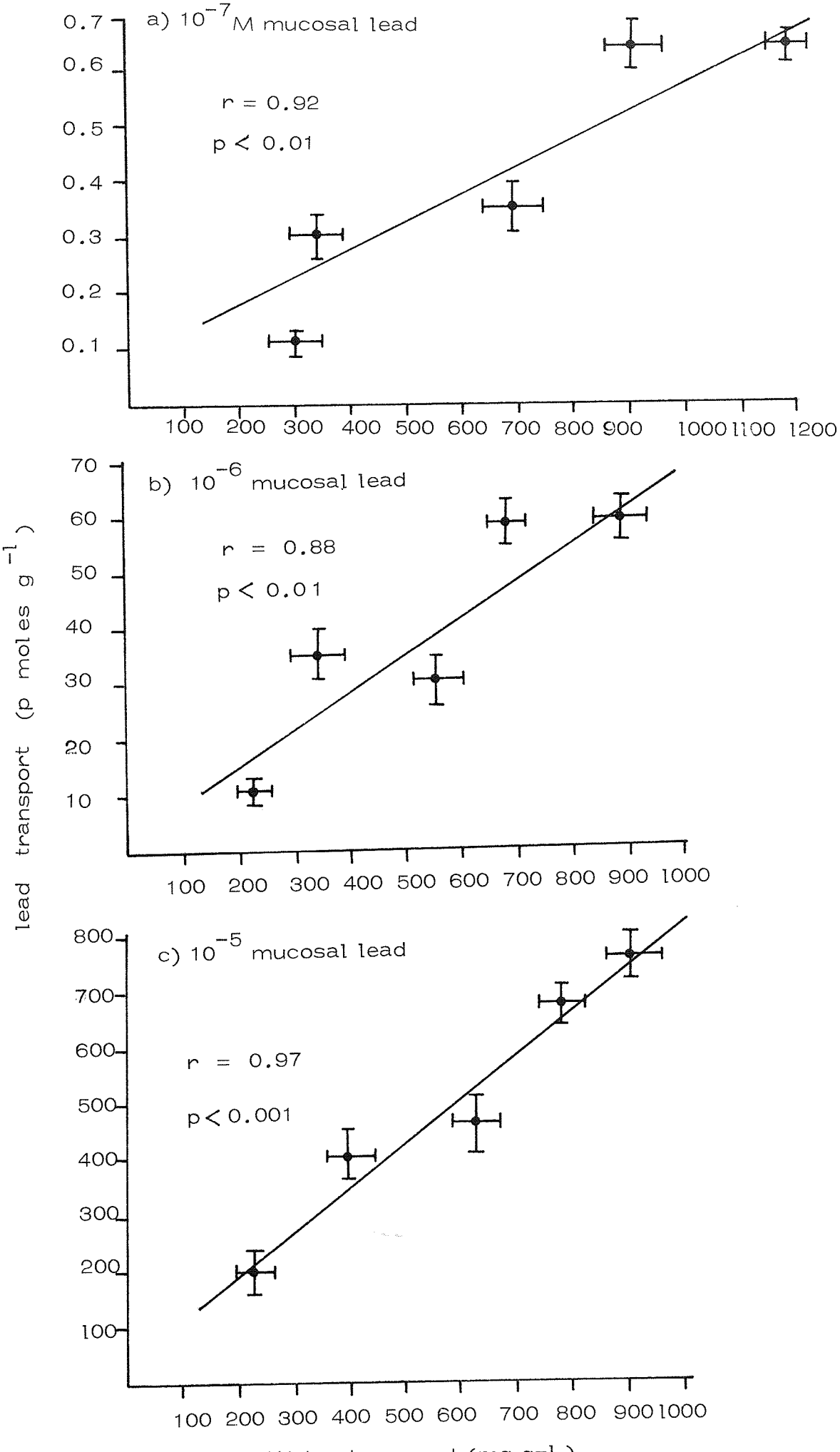
(95% : 5%). Lead uptake and transport was measured as described in Section 2.4.

Lead transport was linear ($r = 0.95$) but significantly slower ($p < 0.01$) than a typical control value, (Figure 4.9 a). The rate of tissue uptake of lead, however, was distinctly different to that demonstrated during incubation under aerobic conditions. Although there was evidence for a rapid initial uptake of lead by the tissue, it accounted for only approximately one-third of the overall uptake. A fast accumulation of lead with time resulted in a significantly greater ($p < 0.001$) amount of lead associated with the intestinal tissue, (Figure 4.9b). These data are at variance with those reported by Coleman (1979), however, their implications will be discussed at length later in this thesis.

4.4. THE EFFECT OF HYPERTONIC MUCOSAL SOLUTIONS ON THE RATE OF LEAD TRANSPORT

Coleman (1979) also demonstrated that under normal conditions there is a reasonable correlation between lead and fluid transport, which led him to conclude that solvent drag was the mechanism responsible for lead transport. Similar correlations ($r = 0.89$) were obtained from the present work, (Figures 4.6 a, 4.6 b and 4.6 c). Further experimentation was conducted to determine whether lead and fluid transport had a causative relationship,

Figure 4.6 The correlation between lead and water transport



conditions were therefore created whereby fluid movement was reduced or reversed.

Everted sacs were incubated at 37°C for 30 minutes in K.H.B. buffer that contained lead acetate (10^{-6}M), ^{203}Pb, and mannitol (100 or 200 mM), and gassed with O_2 (95%) CO_2 (5%). The tissue uptake and transport of lead was measured as described in Section 2.4.

The presence of 100 mM mannitol on the mucosal side of the preparation caused a 90% reduction in the rate of fluid transport, but had no significant effect on the rate of lead transport, (Table 4.2). Further, when the overall direction of fluid movement was reversed by the presence of 200 mM mannitol, i.e. bulk fluid movement was from the serosal to the mucosal sides of the preparation, there was also no significant effect on the rate of lead transport. Neither condition significantly affected the rate of lead uptake by the intestinal tissue. The experimental data clearly demonstrate that although a good correlation exists between lead and fluid movements, the transport of lead is not dependent on solvent drag.

4.5. INVESTIGATIONS INTO THE TENACITY OF THE LEAD INTERACTION WITH INTESTINAL TISSUE

Coleman (1979) postulated the formation of a chemical bond between lead and surface phosphate groups that cause the lead taken

TABLE 4.2. THE EFFECT OF MUCOSAL MANNITOL ON THE TRANSPORT AND TISSUE UPTAKE OF LEAD

	CONTROL	100 mM MANNITOL	200 mM MANNITOL
LEAD TRANSPORT (p moles g^{-1})	24.28 $\pm$ 2.38	27.07 $\pm$ 3.46	22.84 $\pm$ 3.03
TISSUE LEAD UPTAKE (n moles g^{-1})	3.60 $\pm$ 0.71	2.65 $\pm$ 0.67	2.84 $\pm$ 0.29
WATER TRANSPORT (mg g^{-1})	416 $\pm$ 38	40 $\pm$ 17 $p<0.001$	-85 $\pm$ 24 $p<0.001$
TISSUE WATER UPTAKE (mg g^{-1})	554 $\pm$ 38	519 $\pm$ 26	344 $\pm$ 26 $p<0.05$

Each value is the mean of six experimental observations $\pm$ standard error of the mean

up to be strongly bound to the tissue surface. A series of tissue-washing experiments were conducted to assess the extent of tissue lead binding at lead concentrations in excess of one thousand fold higher than those used by Coleman.

Everted sacs were incubated at 37^{o}C for 30 minutes in Tris buffer that contained various concentrations of lead acetate (10^{-6}M - 10^{-1} M) and ^{203}Pb, and gassed with oxygen (100%). The sacs were transferred to Tris buffer that contained various concentrations of lead acetate (10^{-6}M - 10^{-1}M) but no ^{203}Pb, and further incubated for 20 minutes. The uptake and transport of lead was measured as described in Section 2.4.

When preincubated intestinal tissue was washed in Tris buffer that contained non-radiolabelled lead at concentrations up to 10^{-2}M, approximately 20% of the radiolabelled lead associated with the tissue was removed during the intial 20 minutes of washing, and very little removed thereafter. However, when preincubated tissue was washed in Tris buffer that contained non-radiolabelled lead at a concentration of 10^{-1}M, approximately 60% of the radiolabelled lead associated with the tissue was removed during the initial 20 minutes of washing, (Table 4.3). Figure 4.7 demonstrates that the intestinal tissue has a limited capacity to tenaciously bind lead. As estimation of the lead-binding capacity of the intestinal tissue is given in Table 4.4 as approximately 0.041 mmoles per gram of tissue

Table 4.3. THE EFFECT OF PREINCUBATION IN LEAD (RADIO LABELLED) AND SUBSEQUENT WASHING IN NON-RADIOLABELLED LEAD UPON THE TISSUE UPTAKE OF LEAD

Mucosal lead concentration (M)	Tissue lead uptake (n moles)	Amount of lead removed (n moles)	Percentage lead removed (%)
10^{-6}	1.5 $\pm$ 0.05	0.40 $\pm$ 0.004	20.20
10^{-5}	16.11 $\pm$ 0.89	4.07 $\pm$ 0.17	20.20
10^{-4}	158.38 $\pm$ 4.8	38.09 $\pm$ 1.60	19.38
10^{-3}	1,867 $\pm$ 59	357 $\pm$ 17.8	16.12
10^{-2}	13,398 $\pm$ 451	3,536 $\pm$ 53.6	20.88
10^{-1}	31,398 $\pm$ 1699	48,282 $\pm$ 1,006	60.59

Each value is the mean of six experimental observations $\pm$ standard error of the mean

Table 4.4. Estimation of the lead binding capacity of intestinal tissue

Mucosal lead concentration/M	% lead taken up by ½ gram of tissue	Amount of lead taken up /n moles	Amount of lead bound after washing / n moles
10^{-6}	18.4	1.84	1.46
10^{-5}	22.9	2.29×10^{1}	1.83×10^{1}
10^{-4}	15.4	1.54×10^{2}	1.23×10^{2}
10^{-3}	16.5	1.65×10^{3}	1.38×10^{3}
10^{-2}	17.2	1.72×10^{4}	1.36×10^{4}
10^{-1}	5.3	5.30×10^{4}	2.09×10^{4}

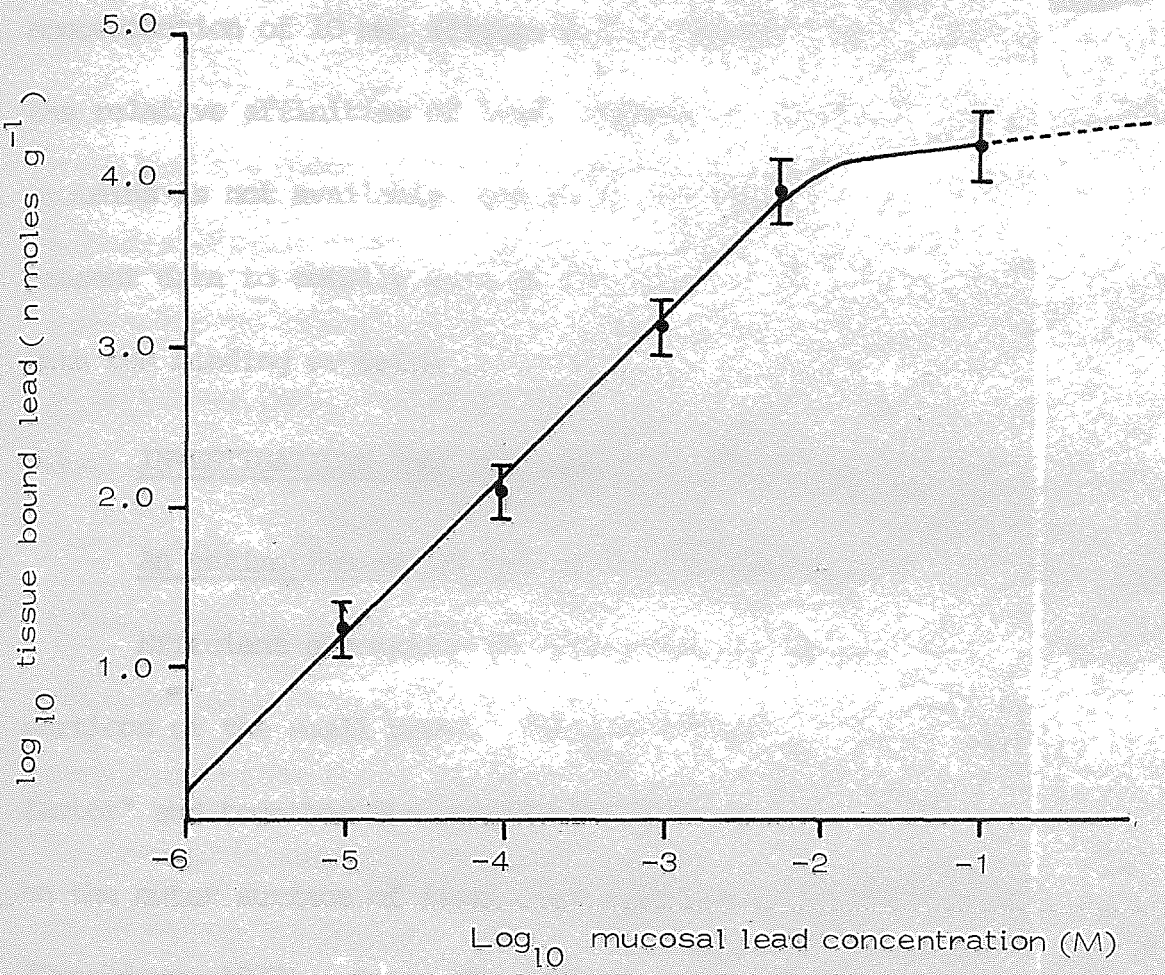
At concentration of 10^{-1} M mucosal lead only 5.3% of the lead available is taken up by the intestinal tissue.

Of this amount of lead 60.59% was washed off over a period of 20 minutes, therefore;

$$\frac{39.41}{100} \times 53000 \text{ n moles of lead were bound}$$

per ½ gram of intestinal tissue = 0.041 m moles g^{-1}

Figure 4.7. The tissue binding of lead after incubation and washing



Each point is the mean value of six experimental observations ± standard error of the mean

The saturation of calcium-bind proteins has been reported to occur at a luminal calcium concentration of 3.5 mM (Kenny, 1975), and iron-binding proteins at 0.45 mM (Cox and Peters, 1979). The process of lead uptake however, is saturated at the higher luminal lead concentration of 10 mM, (figure 4.7). Unfortunately, data concerning the relative affinities of lead, calcium and iron for their respective proteins is not available, and it is not therefore possible for the present data to totally exclude the possibility that lead interacts with the binding proteins.

4.6. INVESTIGATIONS INTO THE DISTRIBUTION OF ABSORBED LEAD

An estimation of the extracellular fluid space

Efficient absorption of cobalamins occurs only from distal portions of the small bowel. Cobalamin binds to an "intrinsic factor" and together the complex binds to specific receptors located on the outer surface of ileal, but not jejunal microvillous membranes, (Donaldson, 1977). Since the jejunum has no receptor sites for cabalamins, they do not enter cells. They are also too large to enter the lateral intercellular spaces, hence they have been used as a probe molecule to assess the extent of the mucosal extracellular fluid space, (Cox and Peters, 1979).

Everted sacs were incubated at 37^{o}C for various times (10-60 minutes) in K.H.B. buffer that contained (^{57}Co)-cyanocobalamin

(10 μCi ug^{-1}, cyanocobalamin concentration 5 nM, Radiochemical Centre, Amersham), and gassed with O_2 (95%) CO_2 (5%). The tissue and 200 μl serosal compartment fluid samples were measured for radioactivity as described in Section 2.4.

The data (Figure 4.8) demonstrated a rapid movement of fluid into the mucosal extracellular spaces, which ceased after 20 minutes of incubation. A value for the mucosal extracellular fluid space for rat jejunum was estimated from Figure 4.8 to be 290 ± 45 μl per gram (wet weight) of tissue. This value compares well with values obtained by other workers, (Jackson et al., 1970 and Cox and Peters, 1979). If it is assumed that no interaction occurs between lead and the intestinal tissue, then the amount of lead that could be contained within the mucosal extracellular fluid space would only account for 8% of the total lead taken up by the tissue in 30 minutes of incubation, therefore, an accumulation of lead must occur on or within the tissue.

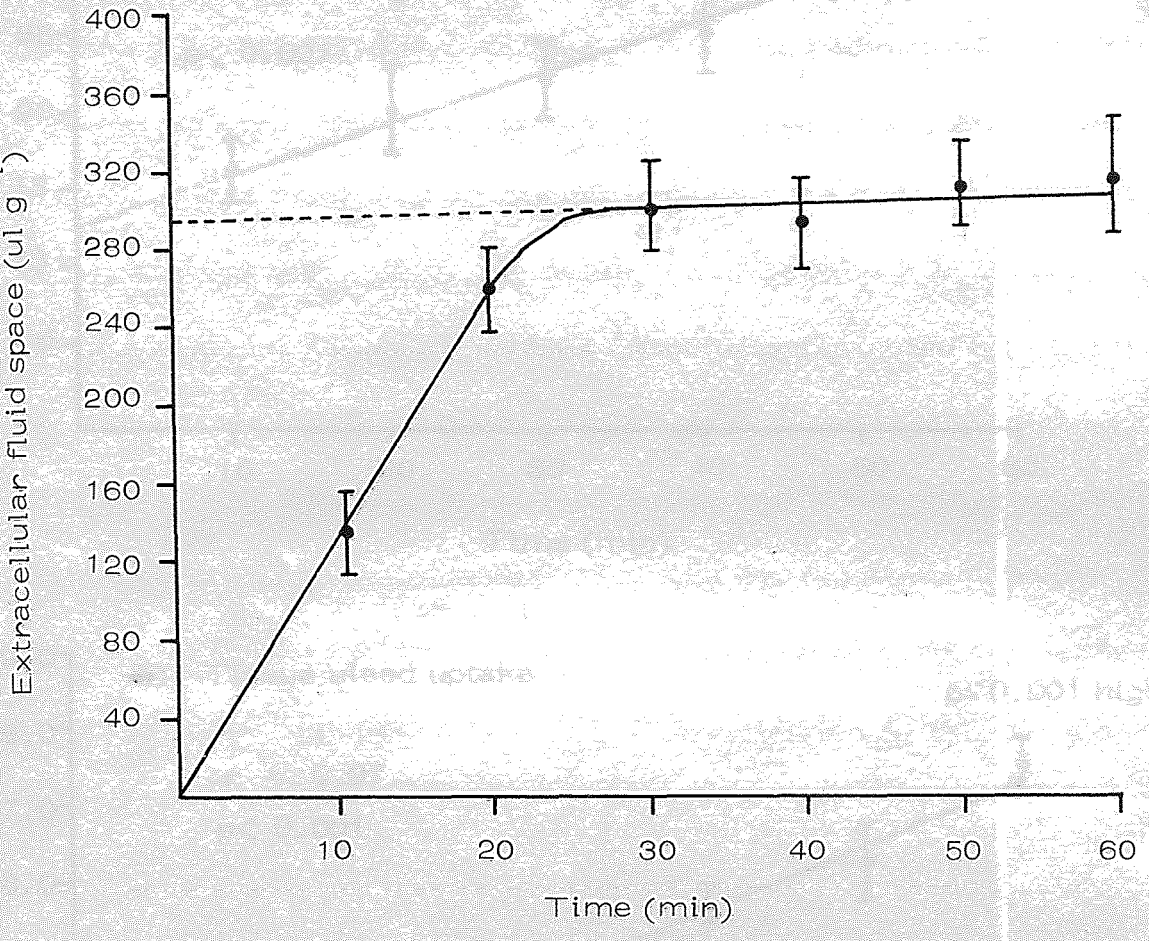
The distribution of lead in mucosal scrapes

The jejunal region of the rat small intestine can be conveniently divided into two parts,

1) the mucosal surface containing the villi, and

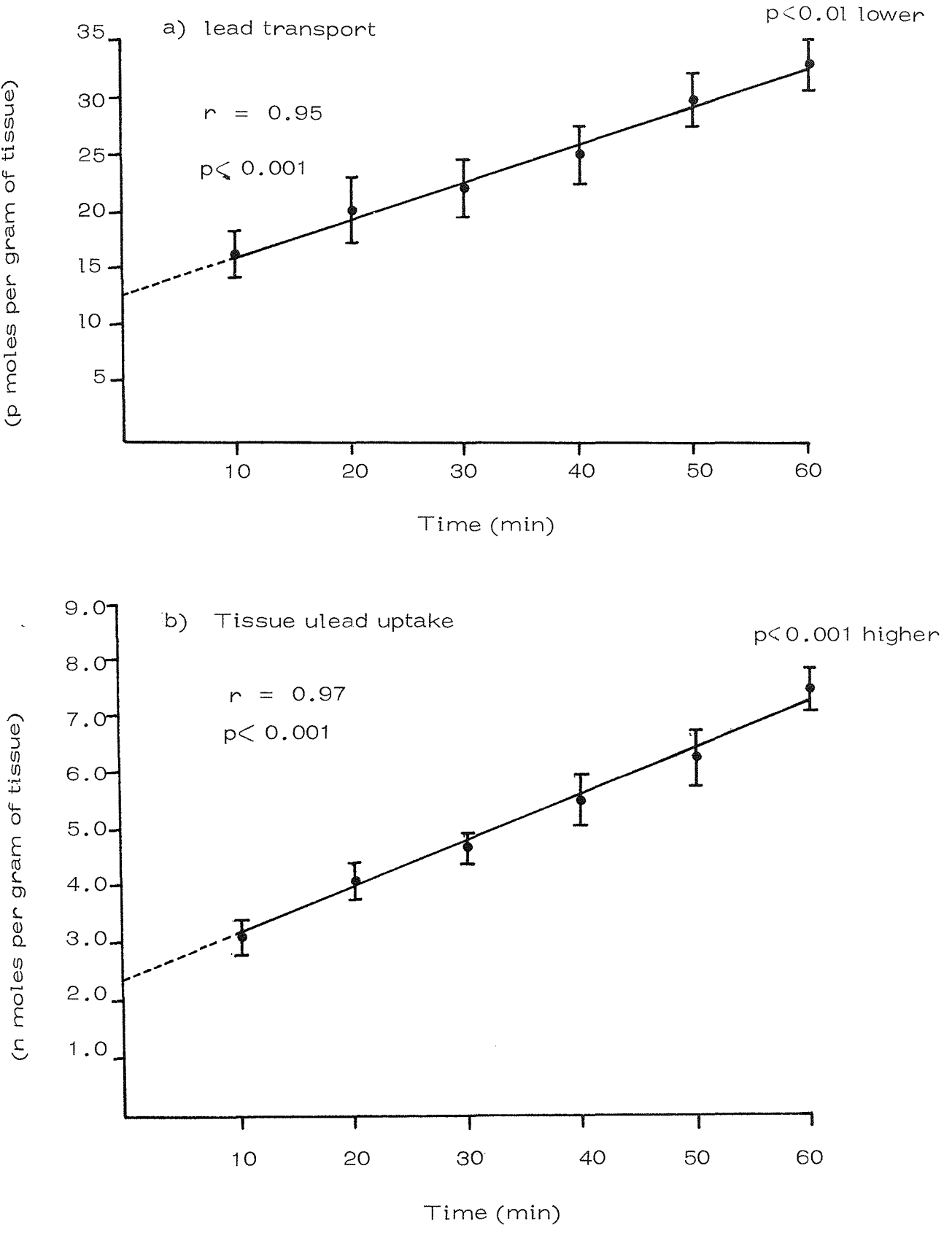
2) the muscularis mucosa containing the underlying blood and lymphatic vessels and muscle layers.

Figure 4.8. Determination of the mucosal extracellular fluid space using (^{57}Co)- cyanocobalamin as an extracellular space marker



Each point is the mean value of six experimental observations ± standard error of the mean

Figure 4.9. The effect of incubation under conditions of anoxia on the rate of transport and uptake of lead



Each point is the mean value of six experimental observations $\pm$ standard error of the mean

Everted sacs were incubated at 37°C for various periods of time (10–60 minutes) in K.H.B. buffer that contained lead acetate (10^{-6}M) and ^{203}Pb, and gassed with O_2 (95%) CO_2 (5%). After incubation the serosal fluid was drained and mucosal scrapings collected as described in Section 2.9. The radioactivity associated with the scraped material, the remaining sac and the serosal compartment fluid was measured as described in Section 2.4. Plates 3 and 4, stained with P.A.S. as described in Section 2.8, demonstrate that the mucosal scrape procedure effectively separated the villi from the muscularis mucosa region. After 10 minutes of incubation, the lead associated with the tissue was distributed between the mucosal epithelial cells and the muscular layers of the muscularis mucosa in approximately the ratio 4:1. This ratio was substantially unchanged at longer incubation periods, (Table 4.5), indicating that the majority of lead taken up is associated with the epithelium.

Localisation of the lead associated with intestinal tissue preparations

The data presented in Section 4.1. demonstrated that a relatively large and rapid uptake of lead by the intestinal tissue occurred in the initial minutes of incubation. It is suggested by data in Section 4.4. that the lead taken up by the tissue is either

1) rapidly transported across the cell membrane and into the cell interior; or

PLATE 3

Section of an everted sac of intestinal tissue stained by P.A.S. reagent as described in Section 2.8.

Magnification approximately x 140

PLATE 4

Section of an everted sac of intestinal tissue after incubation for 60 minutes, and scraped as described in Section 2.9. Tissue was stained by P.A.S. reagent as described in Section 2.8.

Magnification approximately x 140

PLATE 3

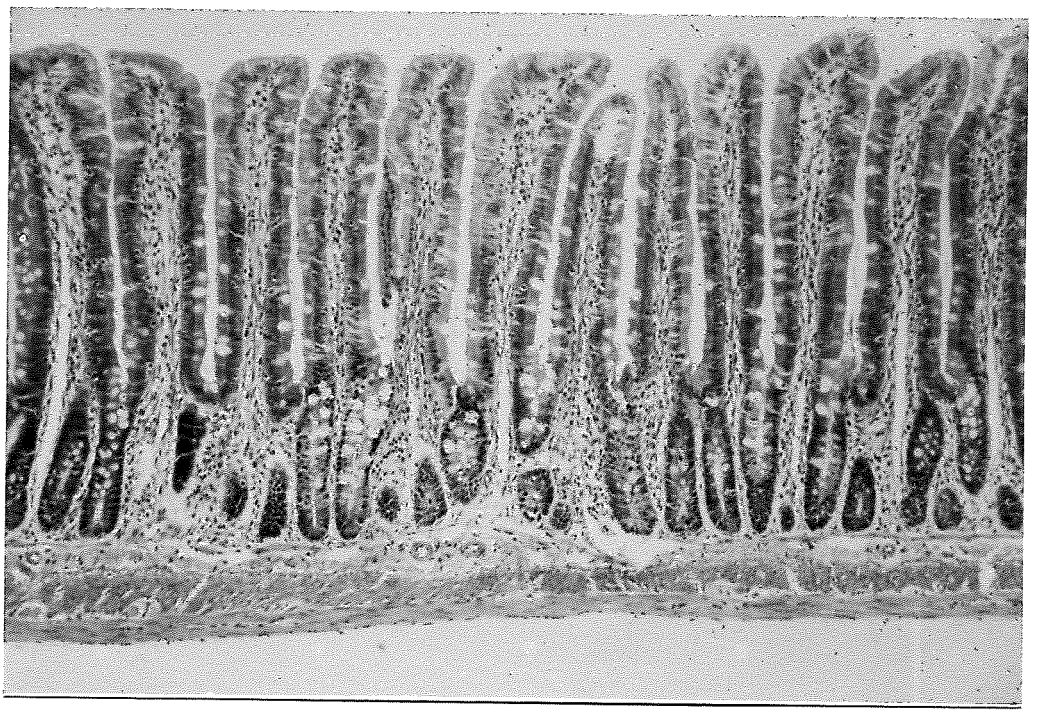


PLATE 4

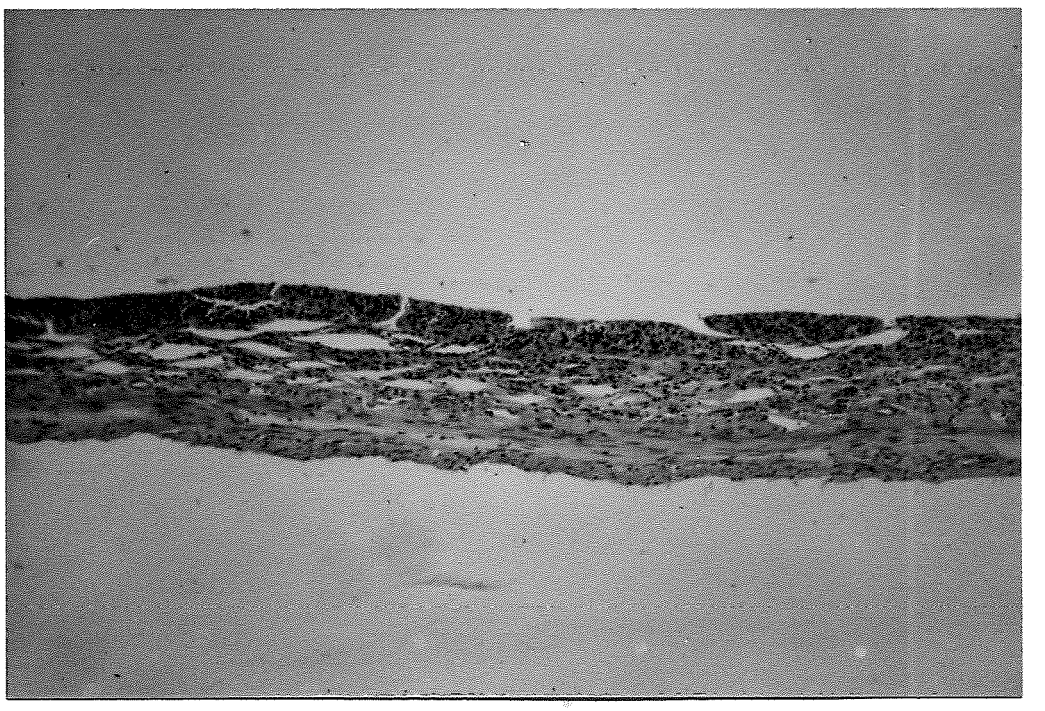


TABLE 4.5. THE DISTRIBUTION OF LEAD AS ASSESSED BY MUCOSAL SCRAPE EXPERIMENTS

INCUBATION TIMES (MINUTES)	LEAD ASSOCIATED WITH SAC (% OF TOTAL)	LEAD ASSOCIATED WITH SCRAPE (% OF TOTAL)
10	17.1 ± 0.8	82.9 ± 0.8
20	21.3 ± 0.9	78.7 ± 0.9
30	21.8 ± 1.1	78.2 ± 1.1
40	15.8 ± 0.7	84.2 ± 0.7
50	16.9 ± 0.8	83.1 ± 0.8
60	22.9 ± 1.1	77.1 ± 1.1

Each value is the mean of six experimental observations ± standard error of the mean

2) adsorbed onto the outer epithelial cell membrane, with very little, if any, entry into epithelial cells. The question of which of the two possibilities is the case may be resolved by the application of histological staining techniques.

Lead may be detected histologically by chemical conversion to a coloured or insoluble compound that can then be visualised and therefore localised. However, in order to allow sufficient accumulation of lead to occur by the preparations, intestinal tissue was incubated in the presence of higher concentrations of lead than would normally be found in the physiological state. The characteristic kinetics of lead uptake by tissue were demonstrated to be the same irrespective of the lead concentration present in the incubation medium. It is therefore assumed that the same process of tissue lead uptake occurs at all lead concentrations investigated, and further that lead localisation when present at high concentrations is representative of the lead-tissue interaction that occurs in the presence of lead at normal concentrations, (i.e. at 10^{-6}M).

In an attempt to localise lead taken up by intestinal tissue, everted sacs were incubated for various times (30-50 minutes) at 37^{o}C in either Saline or Tris buffer that contained various concentrations of lead (10^{-2}M-10^{-1}M), and gassed with O_2 (100%). Sections of the incubated intestinal tissue were fixed and stained by either the chromate, P.A.S. or rhodizonate method as described in

Section 2.9. The appearance of the stained tissue was photographed through a light microscope and presented as plates 5 to 13.

Plates 5, 6 and 7 demonstrate that the presence of lead at high concentrations (10^{-2}M) has no adverse affects on the structural integrity of the tissue preparations. A concentration of stain at the tissue surface is demonstrated by plates 8, 9, 10, 11, 12 and 13, and stain is further shown to extend along the whole length of the villi, (plates 10, 11, 12 and 13). An accumulation of stain is also demonstrated to be present between the epithelial cells, (plate 13), and this may be indicative of the presence of lead in the extracellular spaces. The data presented supports the contention that lead is associated predominantly with the surface of the intestinal epithelium.

4.7. THE EFFECT OF CHELATING AGENTS ON THE RATE OF TISSUE UPTAKE AND TRANSPORT OF LEAD

The experimental investigations reported so far, have used lead which is predominantly in the ionic form. However, in the *in vivo* situation the environment of the intestinal lumen may result in some of the lead being present in a chelated form. The aim of the present investigation is to assess whether the chemical form of lead, (i.e. ionic lead or a chelated lead species) has a significant effect upon the kinetics of lead transfer in the small intestine.

Everted sacs were incubated at 37^{o}C for various periods of

PLATE 5

Section of an everted sac incubated at 37°C for 50 minutes in Tris buffer that contained 10^{-2} M lead acetate. Tissue was stained with P.A.S. reagent as described in Section 2.8. Magnification approximately x 375

PLATE 6

Section of an everted sac incubated at 37°C for 50 minutes in Tris buffer that contained 10^{-2} M lead acetate. Tissue was stained with P.A.S. reagent as described in Section 2.8. Magnification approximately x 375

PLATE 7

Section of an everted sac incubated at 37°C for 50 minutes in Tris buffer that contained 10^{-2}M lead acetate. Tissue was stained with P.A.S reagent as described in Section 2.8. Magnification approximately x 900

PLATE 5

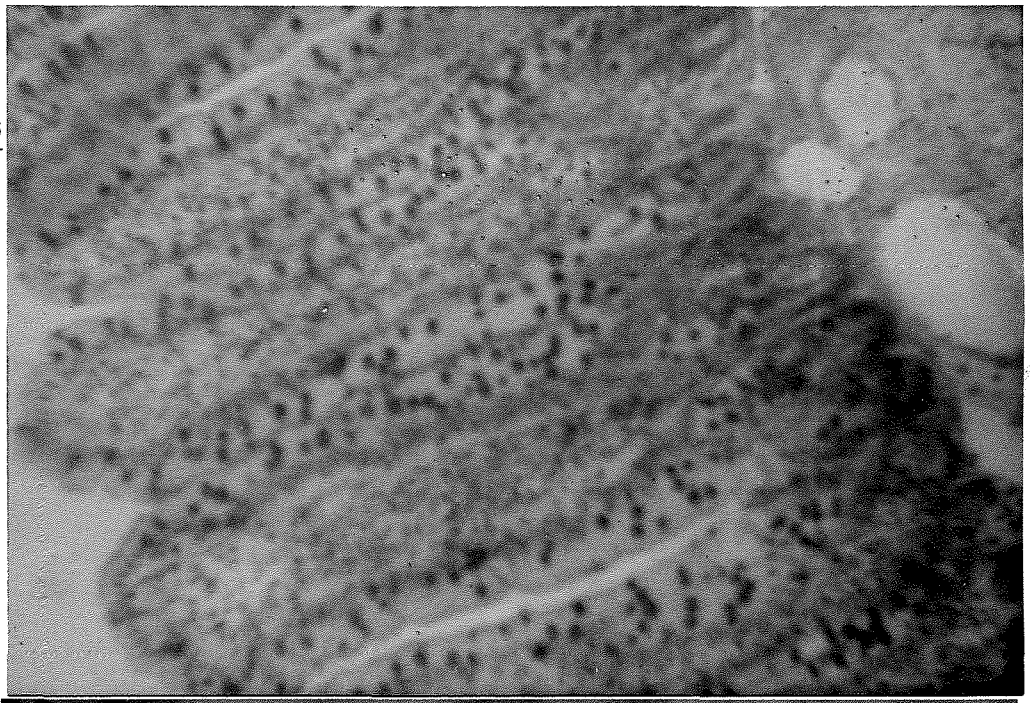


PLATE 6

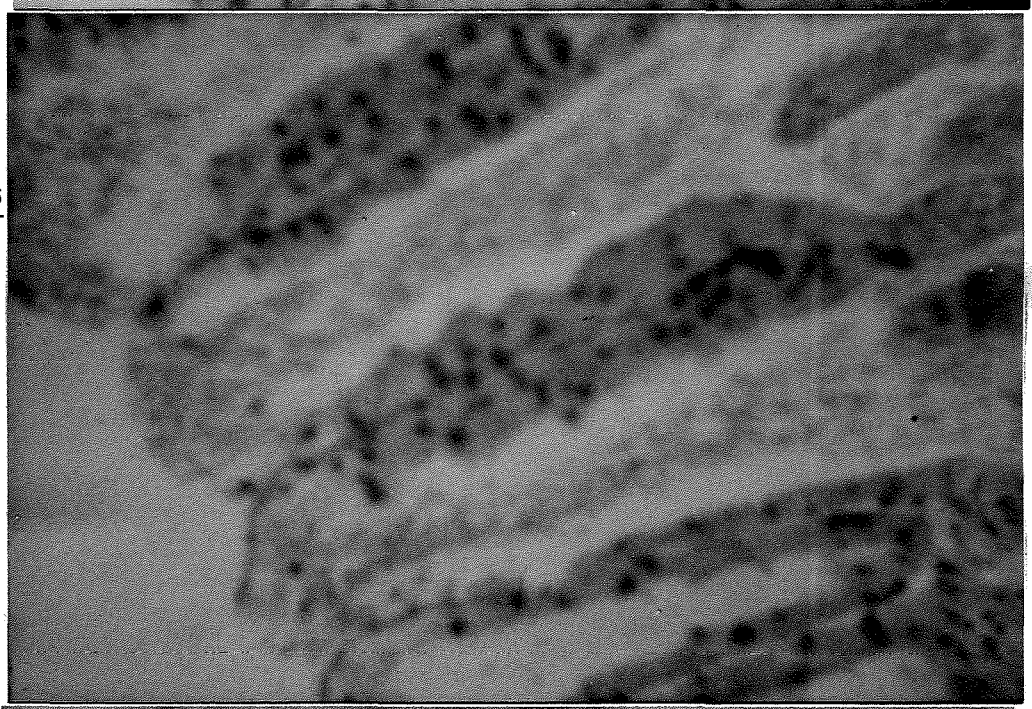


PLATE 7

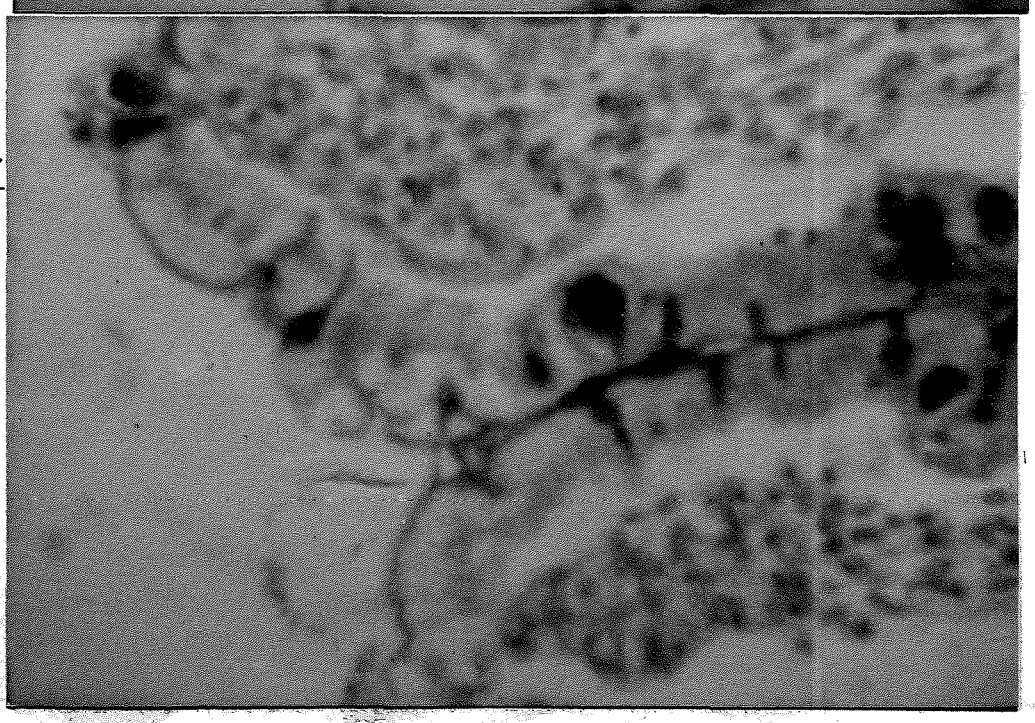


PLATE 8

Section of an everted sac incubated at 37°C for 30 minutes in Tris buffer that contained 10^{-1} M lead acetate. Tissue stained by the chromate method as described in Section 2.8. Magnification approximately x 900

PLATE 9

Section of an everted sac incubated at 37°C for 30 minutes in Tris buffer that contained 10^{-1} M lead acetate. Tissue stained by the Rhodizonate method as described in Section 2.8. Magnification approximately x 900

PLATE 8

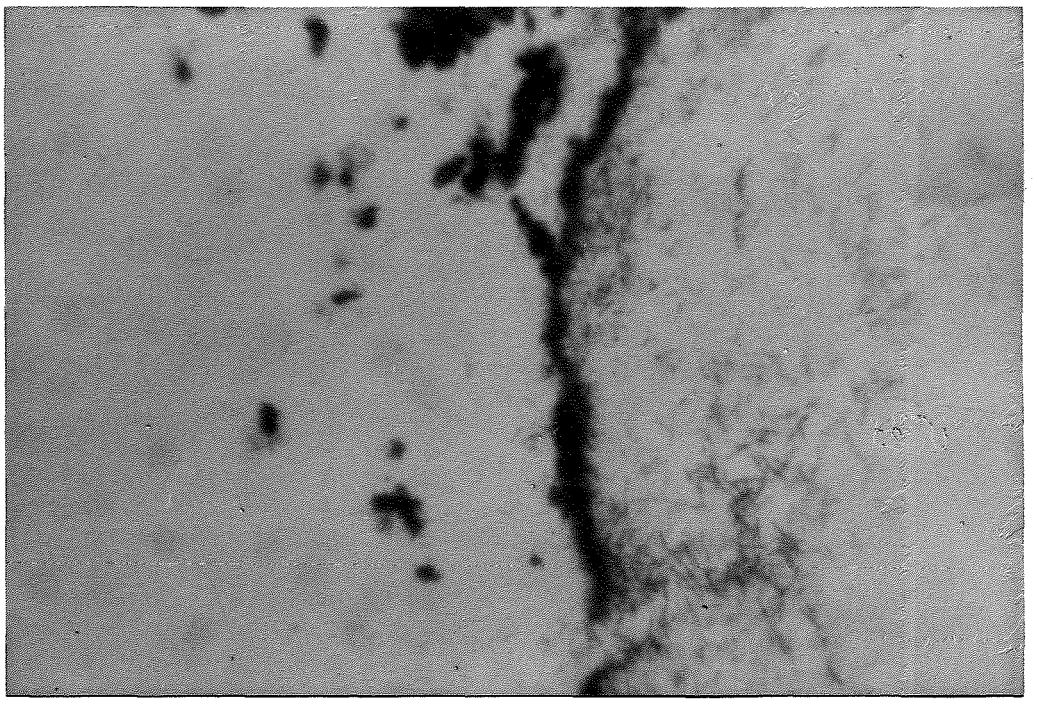


PLATE 9

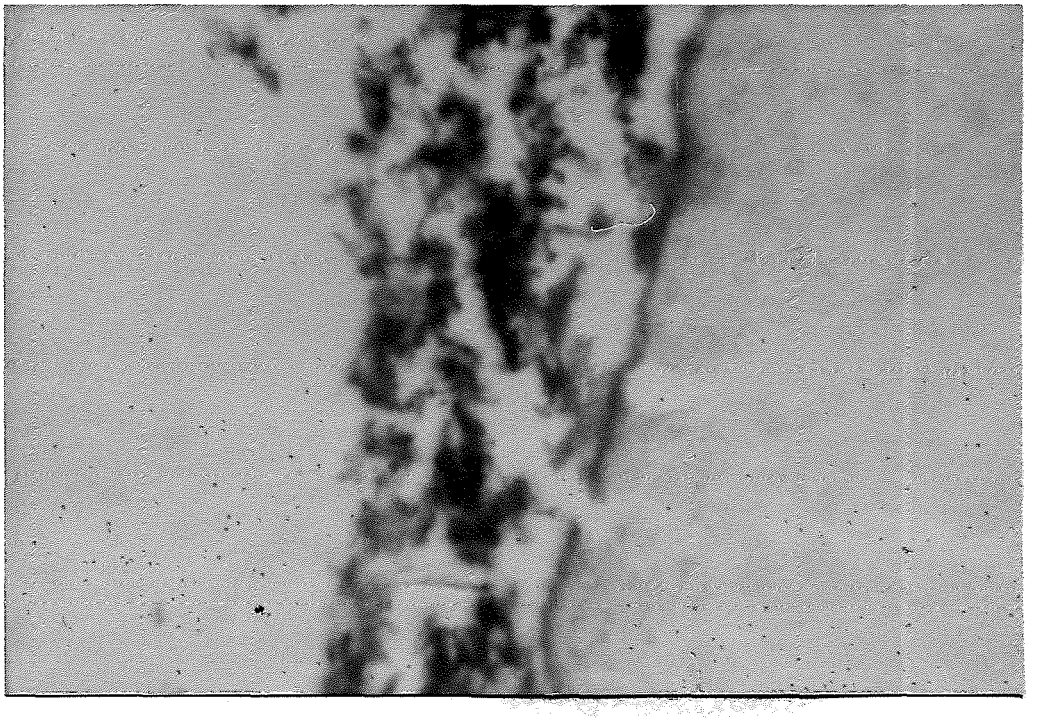


PLATE 10

Section of an everted sac incubated at $37^{o}C$ for 50 minutes in saline buffer that contained 10^{-2} M lead acetate. Tissue stained by the Rhodizonate method as described in Section 2.8.

Magnification approximately x 200

PLATE 11

Section of an everted sac incubated at $37^{o}C$ for 50 minutes in saline buffer that contained 10^{-2} M lead acetate. Tissue stained by the Rhodizonate method as described in Section 2.8.

Magnification approximately x 900

PLATE 10

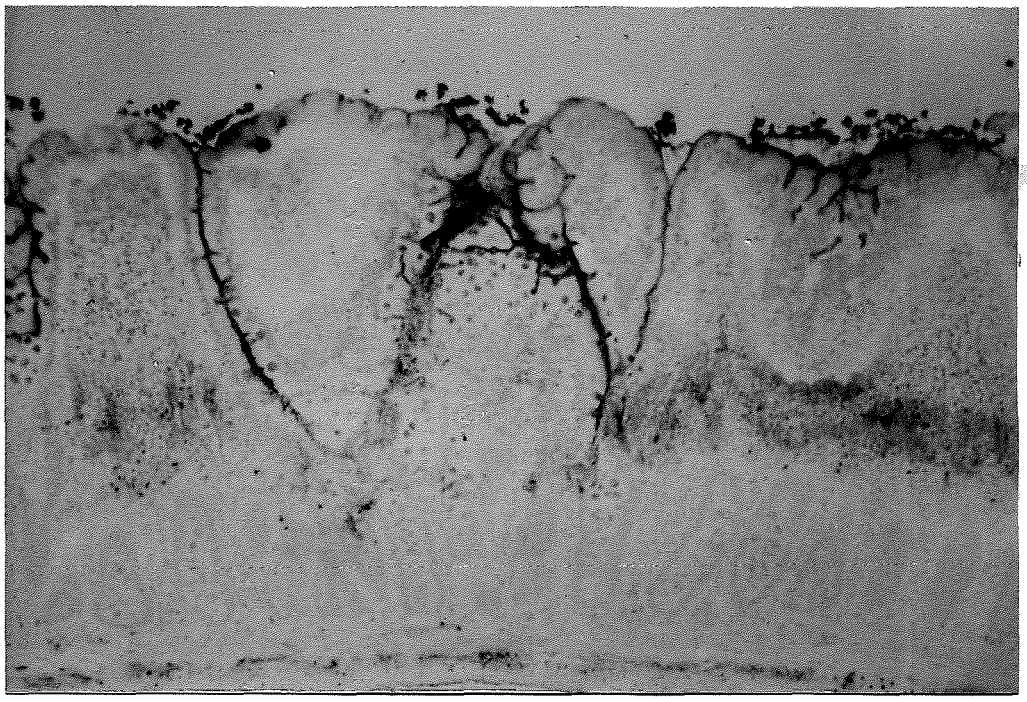


PLATE 11

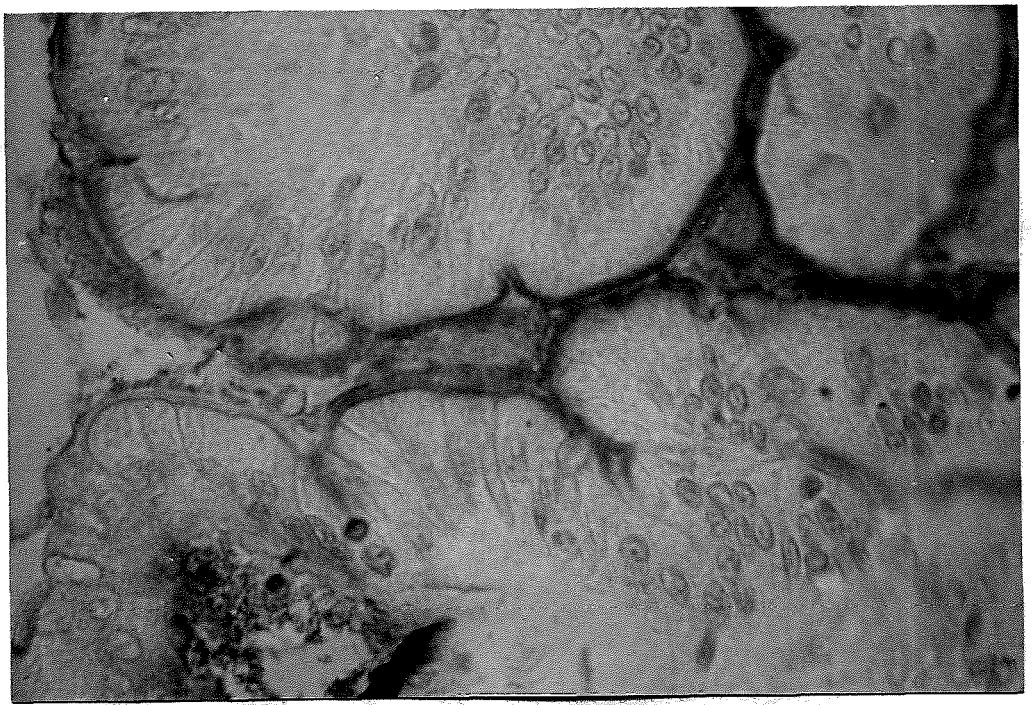


PLATE 12

Section of an everted sac incubated at 37°C for 50 minutes in saline buffer that contained 10^{-2} M lead acetate. Tissue stained by the chromate method as described in Section 2.8.

Magnification approximately x 200

PLATE 13

Section of an everted sac incubated at 37°C for 50 minutes in saline buffer that contained 10^{-2} M lead acetate. Tissue stained by the chromate method as described in Section 2.8.

Magnification approximately x 900

PLATE 12

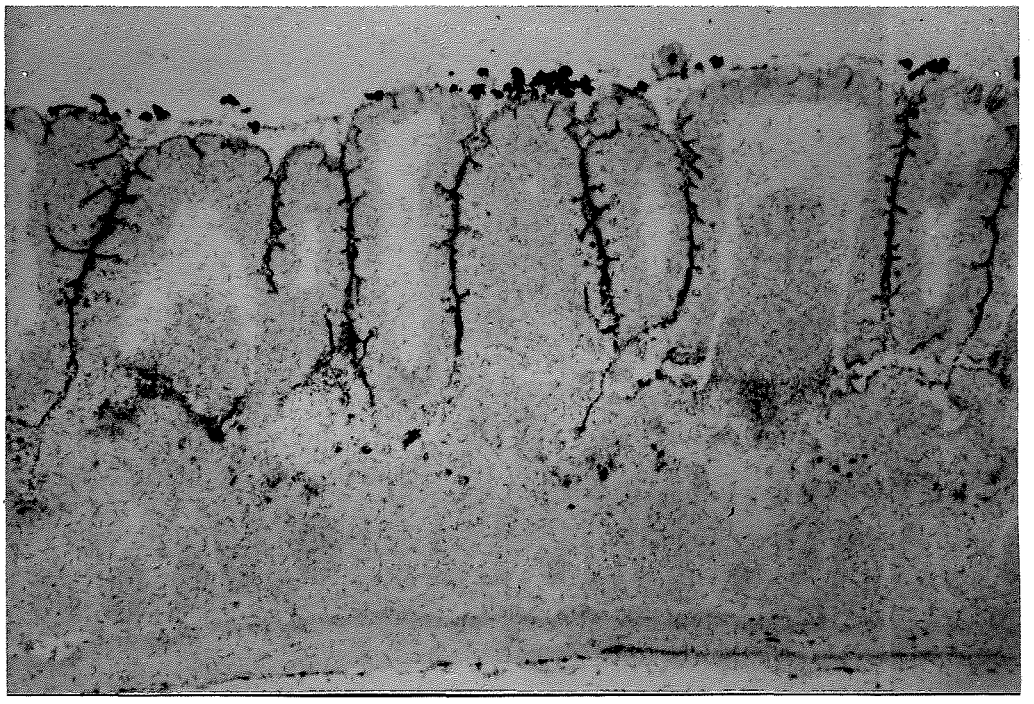
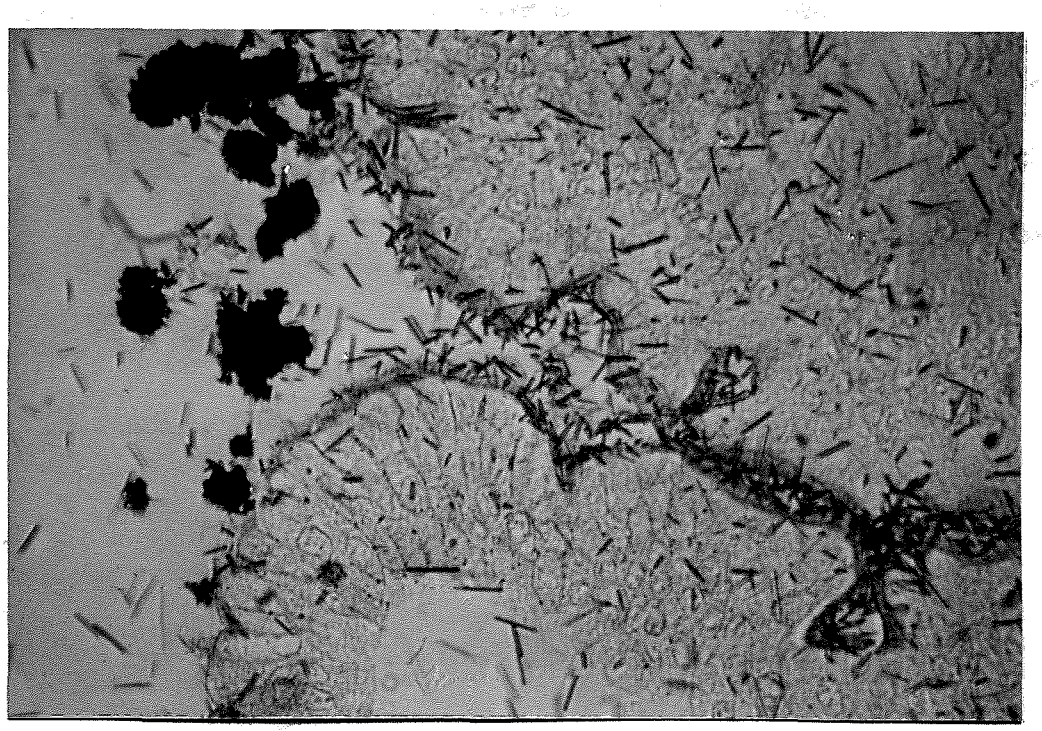


PLATE 13



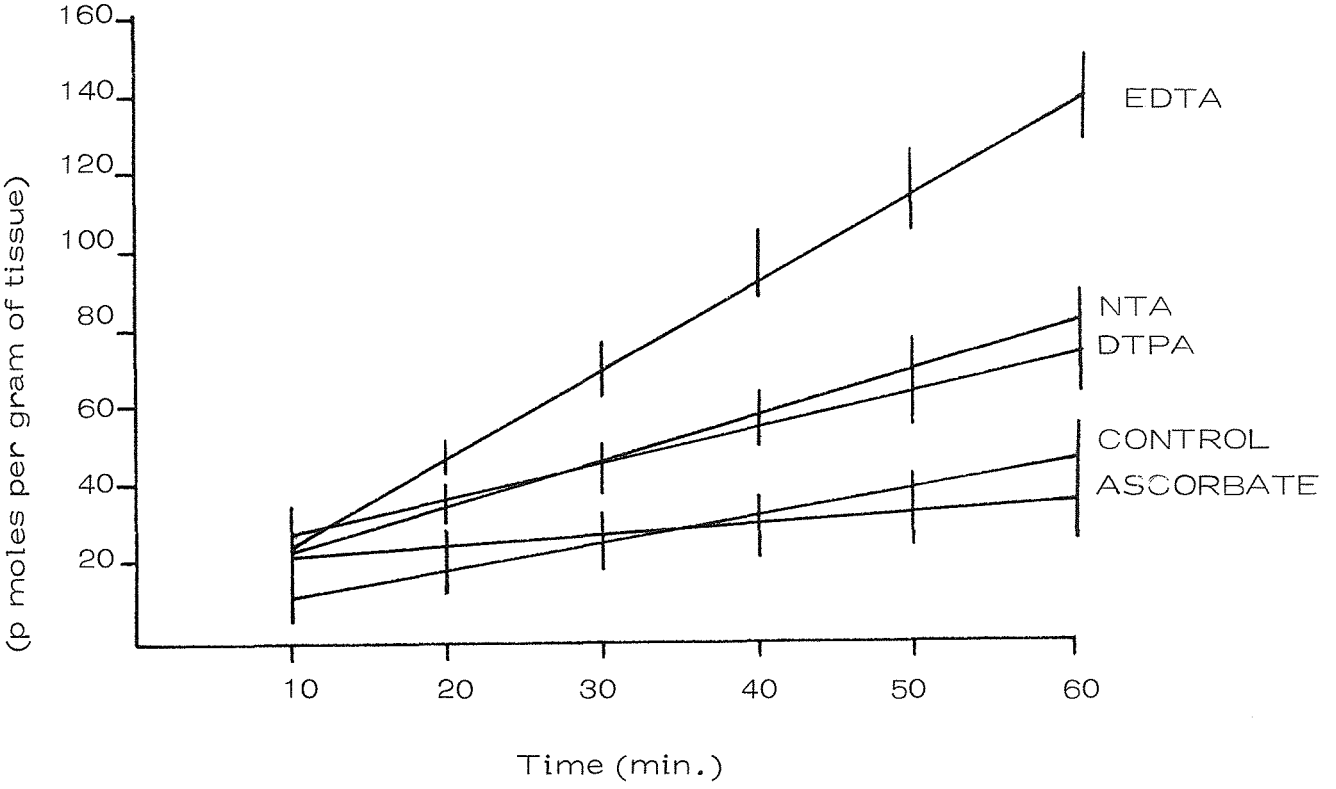
time (10-60 minutes) in K.H.B. buffer that contained either lead acetate (10^{-6}M labelled with ^{203}Pb), or lead ethylenediaminetetra-acetic acid (E.D.T.A), lead nitrilotriacetic acid (N.T.A), lead diethylenetriaminepentaacetic acid (D.T.P.A.) or lead ascorbate, each labelled with ^{203}Pb, and gassed with O_2 (95%) CO_2 (5%). Each of the chelating agents was present at a hundred fold excess over lead (i.e. at 10^{-4}M) to ensure that the complex did not dissociate, (Forth and Rummel, 1975). Lead uptake and transport was measured as described in Section 2.4.

The rate of lead transport was significantly increased when lead was complexed with E.D.T.A. ($p < 0.001$), N.T.A. ($p < 0.001$), D.T.P.A. ($p < 0.05$) but not ascorbate, (Figure 4.10a). The tissue uptake of lead, however, was significantly decreased when lead was complexed with E.D.T.A. ($p < 0.05$), N.T.A. ($p < 0.01$) and D.T.P.A. ($p < 0.001$) but not with ascorbate, (Figure 4.10 b). Some of the data reported by other workers (see Table 1.2, Chapter one) suggest that chelating agents increase the body entry of lead, while others report no effect. Few of the authors comment upon the effect of tissue interaction of lead chelates, and consequently little is known about the mechanism of transport of these species. The data reported here however, demonstrate that the rate of lead transfer may be greatly increased if lead is in a chelated form, and that there may be a greatly reduced tissue interaction of the chelated

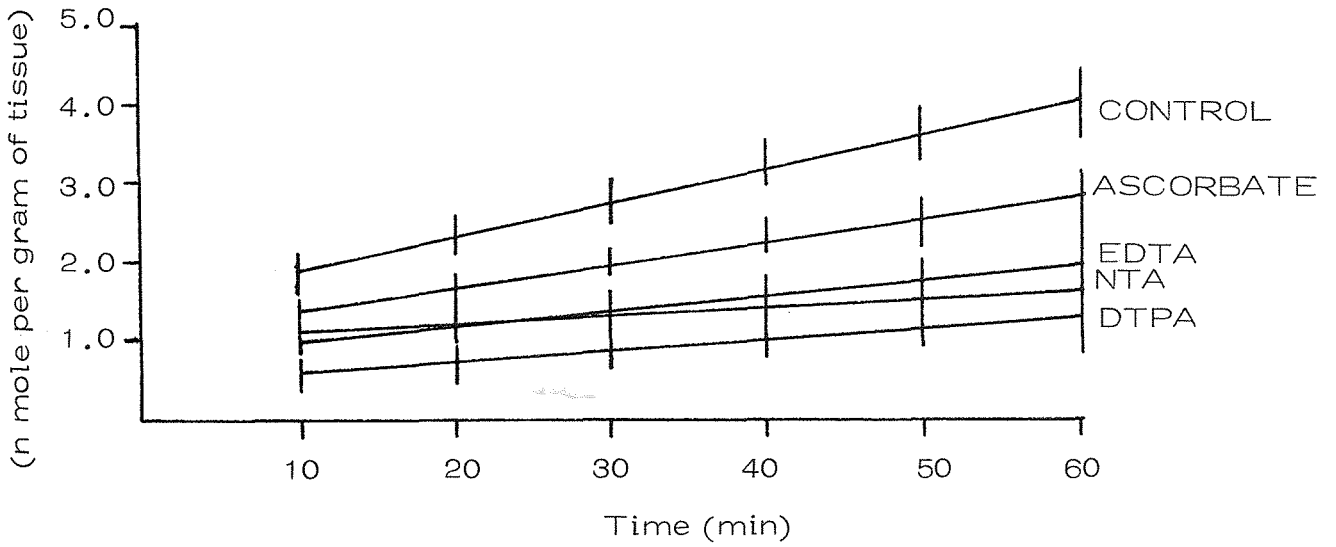
Figure 4.10

A time based investigation of the transport and tissue uptake of certain lead chelates

a) Lead transport



b) Tissue lead uptake



Each value is the mean of six experimental observations ± standard error of the mean

lead species. Both these observations strongly suggest that chelated lead species exhibit different kinetics to those of the lead cation. Further discussion on lead chelates will be given in Section 7.6 of Chaper seven.

4.8. DISCUSSION

The absorption of lead by everted sacs of intestinal tissue demonstrated two distinct phases,

1) an initial rapid uptake of lead by the tissue, with comparatively little further uptake thereafter; and

2) a slow linear concomitant movement of lead to the serosal compartment.

The rate of both processes was proportional to the initial lead concentration of the incubation medium, and neither demonstrated saturation kinetics over the lead concentration range of 10^{-5} - 10^{-2}M. Both processes were demonstrated to be passive. The data are similar with those presented by Coleman (1979) who postulated a surface adsorption phenomen and a slow passive diffusion of lead ions to the serosal compartment.

It has been suggested that lipid membranes are permeable to lead ions (Barltrop and Smith, 1971), and it has been further suggested that transmembrane transport of ions may be facilitated by

membrane proteins, (Singer, 1974). However, the experimental data of a rapid lead uptake cannot be interpreted as cell-entry since the rate of tissue lead uptake is rapid, then comparatively very slow, and this same phenomen is exhibited irrespective of the lead concentration present. The data are more compatable with a tissue surface adsorption process than one of cell permeation, and plates 8-13 confirm this view.

The temperature of incubation, the presence of a metabolic inhibitor and the absence of glucose had no effect on lead uptake or transport, and again confirm the conclusion of Coleman (1979) and Blair et al., (1979). However, the presence of iodoacetate and conditions of anoxia significantly elevated the amount of lead associated with the tissue, and significantly reduced the rate of lead transport. Other workers (Aungst and Fung, 1981) have reported data that stand in contradiction to those reported here, namely that lead transport demonstrates a saturation phenomen at mucosal lead concentrations in excess of 10^{-5}M, and further, anoxic conditions significantly reduce the tissue uptake of lead. The reported saturation of lead transport at approximately 10^{-5}M is a hundred fold lower than what would be expected if the mechanism were, as Aungst and Fung suggest, the same as that for iron or calcium transport. The increase of lead uptake by intestinal tissue under conditions of anoxia and in the presence of iodoacetate may be rationalised by the effect of these conditions on the acid-microclimate.

The acid-microclimate

It has been postulated that an acid-microclimate exists at the surface of the intestine and other tissues, (Blair and Matty, 1974). The acidity is due to the production of protons by external hydrolysis of metabolically produced A.T.P. by the presence of A.T.P-ases in the intestinal brush border, (Noronah and Kesavan, 1979 Forstner et al., 1968). The glycocalyx (see Section 1-3, Chapter one) acts as a barrier to proton diffusion (Pfeiffer, 1981) and assists in maintaining a low surface pH, (approximately 3.5). The acid-microclimate may perform two functions in the process of lead absorption by intestinal tissue. The first is to produce phosphate ions as a bi-product of A.T.P. hydrolysis, that could initiate precipitation of lead phosphate onto the tissue surface. The second function is the generation of protons that would mask negatively charged functional groups present as an integral component of the phospholipid membrane, thus reducing the possible extent of lead cation interaction with the tissue. Any factor that diminishes the acid-microclimate, such as inhibition of glycolysis would cause the tissue surface to become more alkaline (less acidic) and thus more integral negative functional groups would become exposed and available for lead cation interaction. Thus the acid-microclimate may have a role in lead absorption.

The significant reduction in the rate of lead transport under

anoxic conditions (Section 4.3) may be explained in terms of a "see-saw" effect between tissue bound lead and lead present in the intestinal lumen. A large tissue uptake of lead will result in there being less available to be transported across the epithelium, and since the rate of lead transport is proportional to the available lead present in the incubation medium (Section 4.1), the overall rate of transport will be reduced. Thus reduced lead transport under conditions of anoxia or in the presence of iodoacetate is not evidence for an active transport mechanism.

CHAPTER FIVE

ELUCIDATION OF THE POSSIBLE ROUTE OF LEAD ION TRANSPORT

5.1. THE PARACELLULAR ROUTE OF TRANSPORT

It has been observed over many years that the small intestine possesses certain properties that have led investigators to denote it as a 'leaky' epithelium, (Diamond, 1974). Such properties as the ability to quickly dissipate transepithelial ionic gradients, (Schultz, 1977); to allow the rapid movement of water in response to osmotic gradients, (Frömter, 1972); and the discovery of low transepithelial electrical resistances, (Frömter and Diamond, 1972) have characterised its leakiness. It has become accepted that the leakiness of the small intestine cannot be attributed to a specific property of the cell membrane, or to the transport of substances across the epithelial sheet at spaces left when cells are removed during cell-turnover; but rather as a direct consequence of transepithelial paracellular pathways. Such pathways are constituted by the junctional complexes called zonulae occludentes, (the points of cell contact at their apical borders), and the lateral intercellular spaces.

Both Machen et al., (1972) and Whittembury and Rawlins, (1971) have demonstrated that the junctional complexes can be permeated by large ions such as lanthanum. Schultz, (1977 a,b) has demonstrated that the paracellular pathways account for more than 90% of the total transepithelial conductance in 'leaky' (gall-bladder) epithelia.

Coleman, (1979) proposed that lead, in common with the passive movement of electrolytes, traversed the intestinal epithelium via the paracellular route. He investigated the effect of increased buffer acidity, and of reduced calcium on the transport of lead by rat jejunum. He reported that serosal transport of lead was significantly enhanced at a luminal pH of 4.4., and by the absence of calcium in the buffer media. Both results are indicative of tight-junction involvement in the transport mechanism. However, as the effect was achieved by conditions which may be considered extreme, further examination was undertaken using conditions which were more relevant to the physiological status of the membrane. Two approaches were adopted:

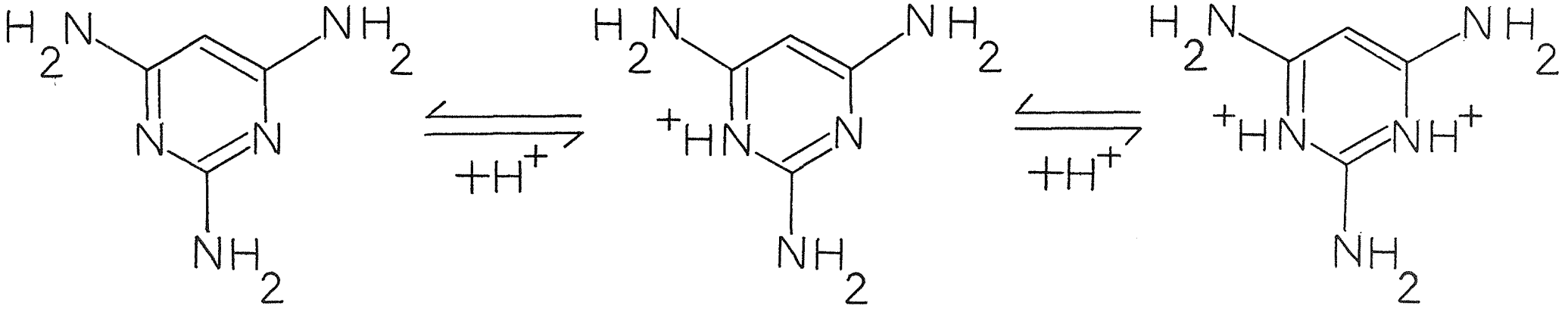
1. an attempt was made to block the tight junctions using 2,4,6-triaminopyrimidine ; and

2. conditions were created that favoured cell swelling and the collapse of lateral extracellular spaces.

5.2. THE EFFECT OF 2,4,6-TRIAMINOPYRIMIDINE ON THE RATE OF LEAD ION TRANSPORT

Moreno (1975) used the organic cation 2,4,6-triamino-pyrimidine (T.A.P., Figure 5.1) to block the passive component of sodium transport across the leaky epithelia of frog small intestine and gall bladder. He postulated that the tight junction was the rate-

Figure 5.1. The structure of 2,4,6-Triaminopyrimidine in the neutral mono and diprotonated forms



Neutral form

Monoprotonated form

diprotonated form

limiting step in paracellular transport.

Studies on the passive ion permeation and selectivity of frog and rabbit gall bladder demonstrated the existence of hydrated channels of approximately 1-2 nm diameter which were cation selective. Within the channels it was suggested that there are ionised acidic groups which are strong H-bond proton acceptors, (Moreno and Diamond, 1974a). T.A.P. has a strong proton donor ability and its dimensions allow it to fit inside the tight junctional channels. Moreno (1975) demonstrated that 80% of sodium conductance could be inhibited by 10 mM T.A.P. If lead ions also traverse the epithelium via a paracellular route then T.A.P. would be expected to significantly decrease the rate of lead transport.

In an attempt to block the tight junctions, everted sacs were incubated for either 30 or 60 minutes at $37^{o}C$ in K.H.B. buffer (pH 6.1) that contained $10^{-6}M$ lead acetate, ^{203}Pb as a tracer and T.A.P. ($10^{-2}M$). The solutions were adjusted to pH 6.1 to mimic the conditions used by Moreno and gives rise to a protonated T.A.P. species, which it is suggested is more effective for the inhibition of sodium flux.

In a separate experiment, everted sacs were incubated for 30 minutes at $37^{o}C$ in K.H.B. buffer (pH 6.1) that contained T.A.P. ($10^{-2}M$), before they were transferred, and incubated for

a further 30 minutes, to the same solution containing lead acetate (10^{-6}M with ^{203}Pb). In all experiments the solutions were gassed with O_2 (95%) CO_2 (5%) throughout the incubations, and the uptake of lead by the intestinal tissue and transport to the serosal compartment measured as described in Section 2-4.

Incubation and preincubation in the presence of T.A.P. produced no significant effect on either the rate of tissue uptake or the rate of transport of lead to the serosal compartment (Table 5-1).

In an endeavour to determine whether T.A.P. affects Na^+ flux, the transmural potential difference was measured in the presence of T.A.P. Jejunal sections of intestinal tissue were everted and arranged to measure transmural potential difference as described in Section 2-6. K.H.B. buffer which contained T.A.P. (10^{-2}M) was placed either on both sides of the preparation or only in the mucosal compartment. The transmural potential difference was measured at 10 minute intervals for a period of one hour. The presence of T.A.P. (10^{-2}M) however, produced no significant effect on the transmural potential difference, (Figure 5.2).

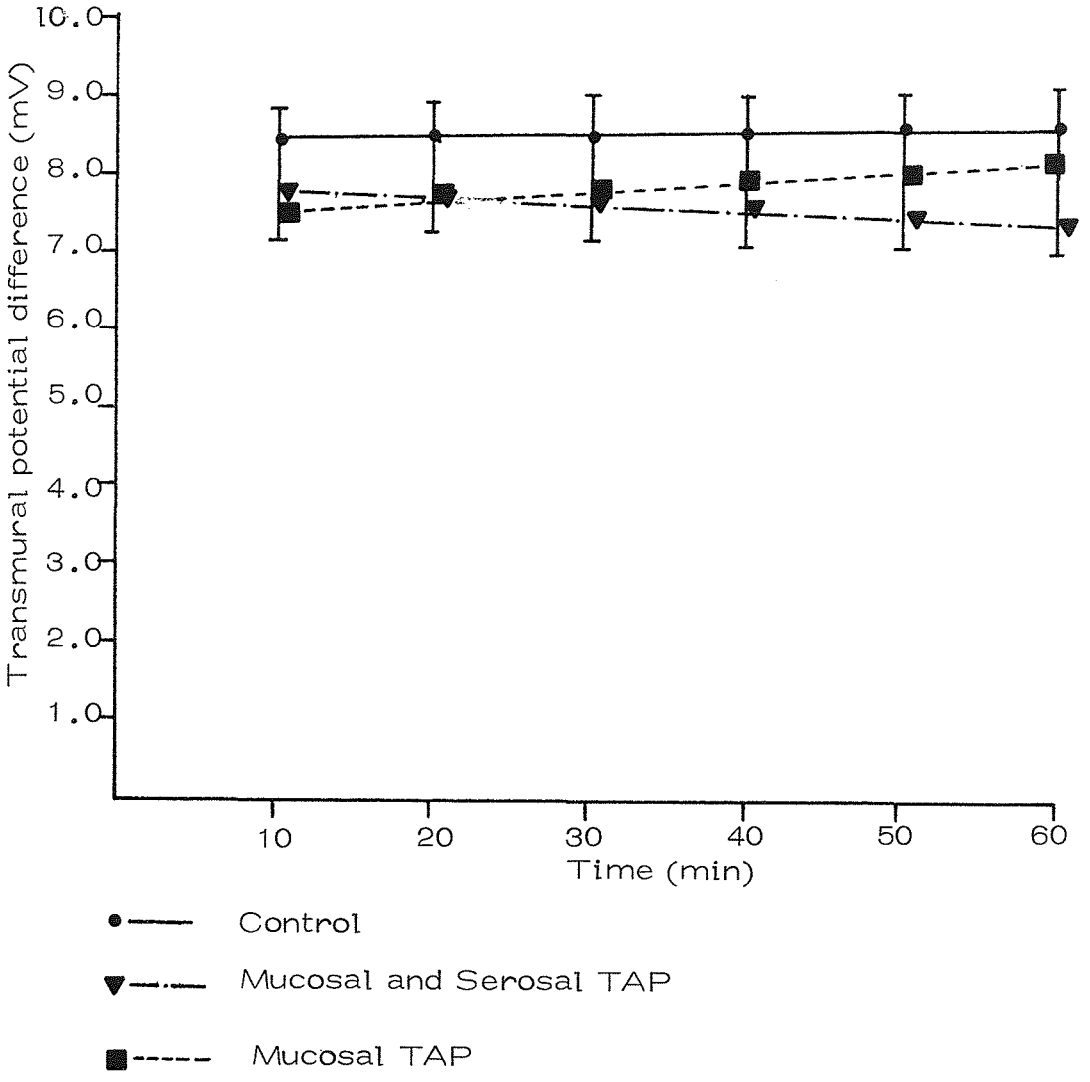
Further investigations were conducted in which the rate of T.A.P. transport was determined spectroscopically. A spectrum scan was taken of a diluted T.A.P sample using a Unicam SP 1700 Ultraviolet Spectrophotometer, (Pye-Unicam Limited, Cambridge).

Table 5.1. The effect of incubation, or preincubation in the presence of 2,4,6-triaminopyrimidine (10^{-2}M) on the rate of lead transport

	30 minutes		60 minutes		Preincubation	
	Serosal (p moles g^{-1})	Tissue (n moles g^{-1})	Serosal (p moles g^{-1})	Tissue (n moles g^{-1})	Serosal (p moles g^{-1})	Tissue (n moles g^{-1})
CONTROL	26.01 ± 3.87	3.14 ± 0.52	45.20 ± 4.31	4.51 ± 0.52	22.89 ± 4.38	2.98 ± 0.80
TEST (10^{-2} M TAP)	N/S 24.09 ± 4.11	N/S 3.09 ± 0.60	N/S 48.31 ± 7.28	N/S 4.63 ± 0.91	N/S 25.55 ± 5.04	N/S 3.13 ± 0.99

Each value is the mean ± S.E.M. of six experimental observations. Preincubation consisted of 30 minutes incubation in K.H.B. buffer containing 10^{-2}M T.A.P, followed by 30 minutes incubation in K.H.B. buffer containing 10^{-2} M T.A.P. and 10^{-6} M lead acetate and ^{203}Pb. N/S = No significant difference between the control value and test value.

Figure 5.2. The variation of transmural potential difference in the presence of 2,4,6-Triaminopyrimidine (10^{-2}M)



Each point is the mean value of six experimental observations $\pm$ standard error of the mean.

Maximum absorbance was attained at 270 nm. A calibration curve for T.A.P. was drawn, (Figure 5.3) and from it the Molar Absorptivity was determined as 1.11×10^{4} moles^{-1} litre. This in conjunction with Beer's law was used to determine the T.A.P. concentration of a sample.

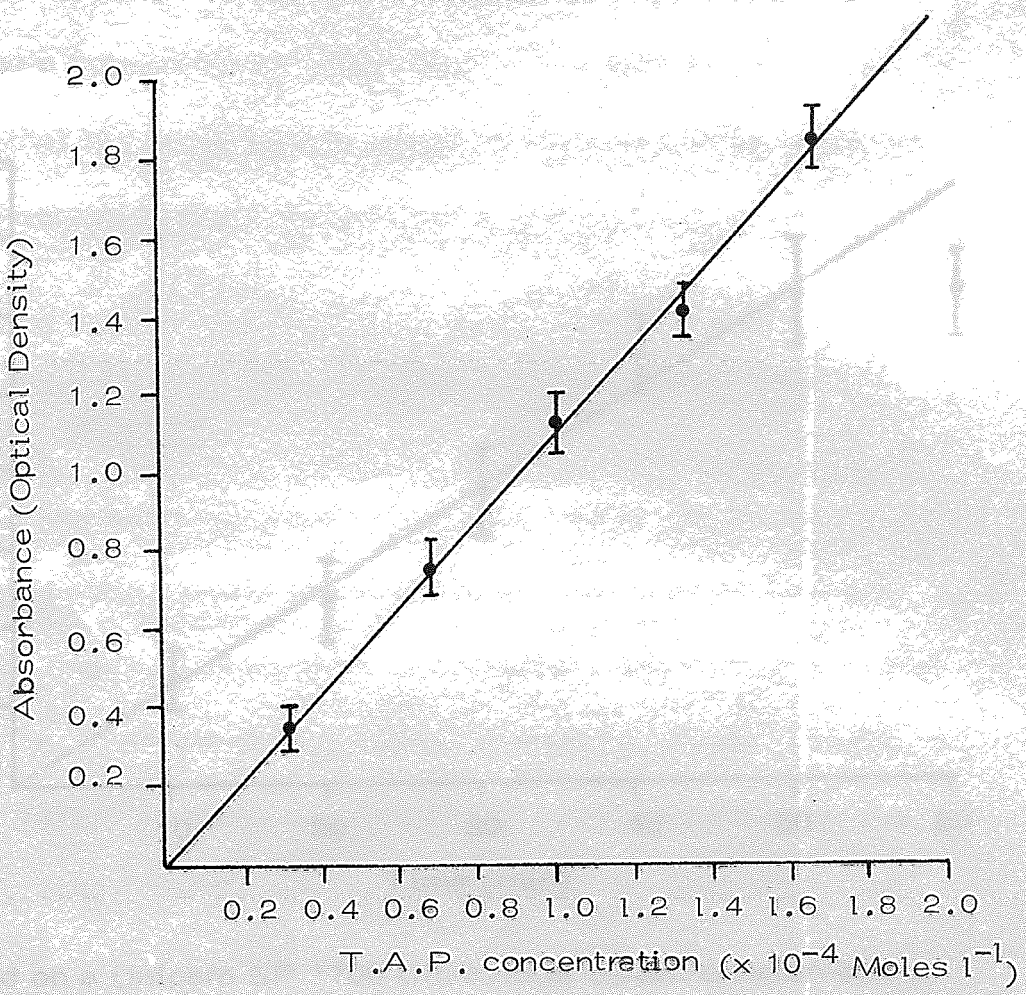
Everted sacs were incubated at 37^{o}C for various periods of time (10-60 minutes) in K.H.B. buffer which contained T.A.P. (10^{-2}M, adjusted to pH 6.1). After incubation a 50 µl sample from the serosal compartment was thoroughly mixed with 2.95 ml of distilled water and put in a quartz cuvette (3 ml), and the optical density at 270 nm determined, as described in Section 2.10. Distilled water was used as a reference.

The data (Figure 5.4) indicates that T.A.P. was transported to the serosal compartment at a slow linear rate over the initial 50 minutes of incubation. The shape of the graph does however, suggest some inhibition to the transport of T.A.P. may occur after 50 minutes, although this is not proven. The rate of transport of T.A.P. to the serosal compartment was approximately 4.5×10^{-7} moles per hour per gram wet weight of tissue.

5.3. DISCUSSION

The fact that there was a slow but continuous arrival of T.A.P. in the serosal compartment suggests that either T.A.P.

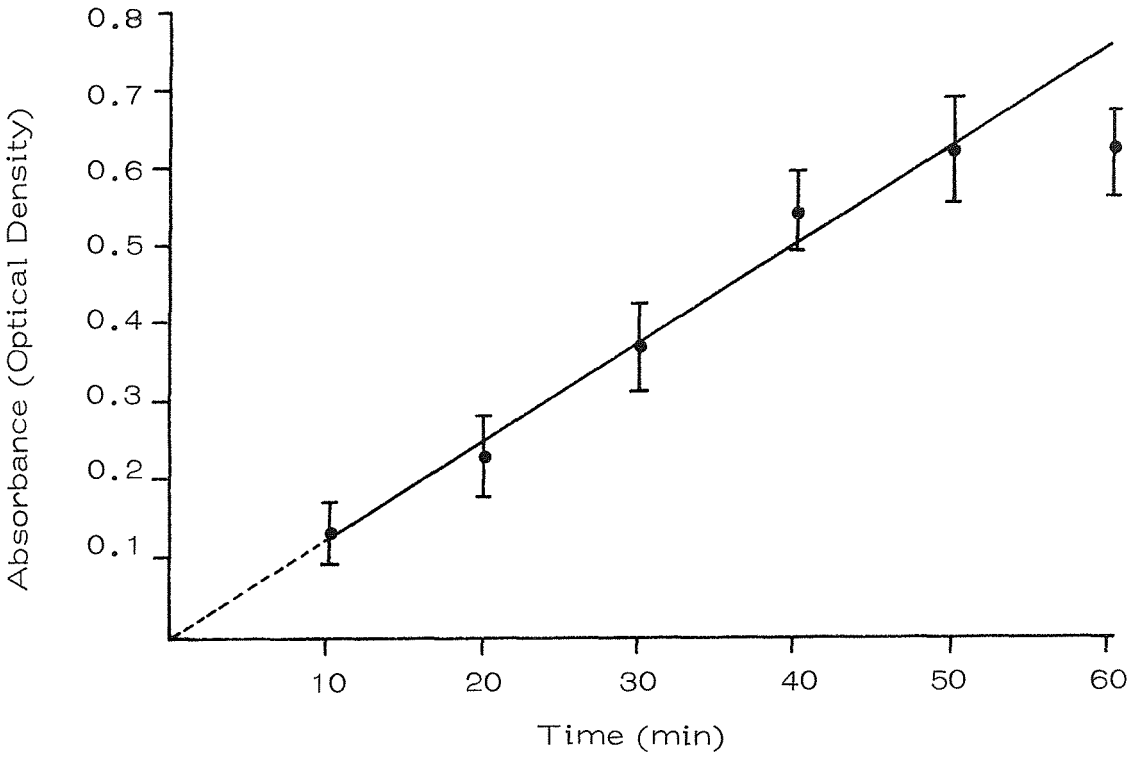
Figure 5.3. The calibration curve for 2,4,6-Triamino-pyrimidine



Optical Density	Concentration (Moles l^{-1})
0.385	3.33×10^{-5}
0.796	6.66×10^{-5}
1.116	1.00×10^{-4}
1.458	1.33×10^{-4}
1.890	1.67×10^{-4}

Each point is the mean value of six experimental observations $\pm$ standard error of the mean

Figure 5.4. The transport of 2,4,6-Triaminopyrimidine measured photocolorimetrically (270 nm)



Measured on a Unicam SP 1700 Ultraviolet Spectrophotometer.

Conditions - Quartz cuvettes, total of 3 ml

Sample size = 0.05 ml with 2.95 ml of distilled water added.

Calibrated by 0.05 ml of 10^{-2} M TAP = Absorbance 2.0

Machine set to absorb at 270 nm

Each point is the mean value of six experimental observations $\pm$ standard error of the mean

was not sufficiently bound, and therefore, not blocking the tight junctions; or that T.A.P. adopts a transcellular mode of transport. Support for the former conclusion comes from the fact that the transmural potential difference was unaffected by the presence of T.A.P. at a concentration of 10^{-2}M. For this reason it is apparent that the conditions obtained by Moreno (1975), have not been reproduced in these experiments.

5.4. OTHER METHODS OF BLOCKING PARACELLULAR TRANSPORT

It has been reported that in both rabbit and human intestine, elevated levels of intracellular cyclic-AMP convert electrolyte and fluid absorption to secretion, and reduce mucosal conductance, (Corbett et al., 1977; Turnberg, 1978; Ilundain and Naftalin, 1979; Naftalin and Simmons, 1979, and Beubler and Lembeck, 1980).

Cyclic-AMP is a relatively inert chemical when applied to intact cells or when injected into intact animals. Among the possible reasons for this are poor penetration through cell membranes, (which seems to be characteristic of phosphorylated compounds in general), and rapid destruction by phosphodiesterase, (Lehninger, 1975). Various analogues and derivatives have been prepared in an attempt to obtain either a better penetration

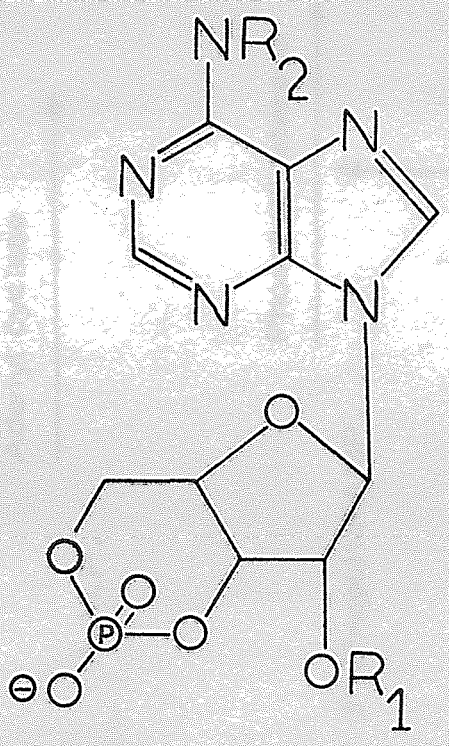
to the interior of the cells or a greater resistance to the action of phosphodiesterase, or both. The derivatives which have so far demonstrated interesting biological properties are those with an acyl group either in position N^6 or 2^1-O or simultaneously in both positions, (Figure 5.5). Certain derivatives have been demonstrated to be more potent than cyclic-AMP when applied to cells, but the reaons for their action is not entirely clear.

The intracellular concentrations of cyclic-AMP at any given time will depend on the relative activities of adenyl cyclase and phosphodiesterase, (Figure 5.6). Normal tissue levels of cyclic-AMP range from 0.1 to 0.5 nmoles per gram of tissue (wet weight), or approximately 0.5 to 2.6 pmoles per mg of protein, (Robison et al., 1971). The intracellular concentration would therefore be approximately 10^{-7}M.

In most tissues, the activity of phosphodiesterase is far greater than that of adenyl cyclase (as measured in cell-free systems). The best known inhibitors of phosphodiesterase are methylxanthines, (Figures 5.7 a-c), which are also very potent compounds in a variety of other systems. For example, in the phosphorylase system alone, in addition to the inhibition of phosphodiesterase, methylxanthines activate phosphorylase phosphatase, and at higher concentrations inhibit phosphorylase and adenyl cyclase. Thus, it is possible that systems involving cyclic-AMP may also be affected by methylxanthines at points other than the

Figure 5.5. The Structure of cyclic-adenosine 3',5'-monophosphoric acid and N^6-2'-O-dibutyryl-cyclic-Adenosine 3',5',-monophosphoric acid

Structure of Adenosine 3',5'-monophosphoric acid ($R_1 = R_2 = H$), abbreviated to cyclic-AMP



Structure of N^6-2'-O-dibutryryl-cyclic-Adenosine 3',5'-monophosphoric acid ($R_1 = R_2 = C_4 H_7 O$), abbreviated to db-cyclic-AMP

Figure 5.6

Effect of Theophylline and Caffeine on the production of cyclic-AMP

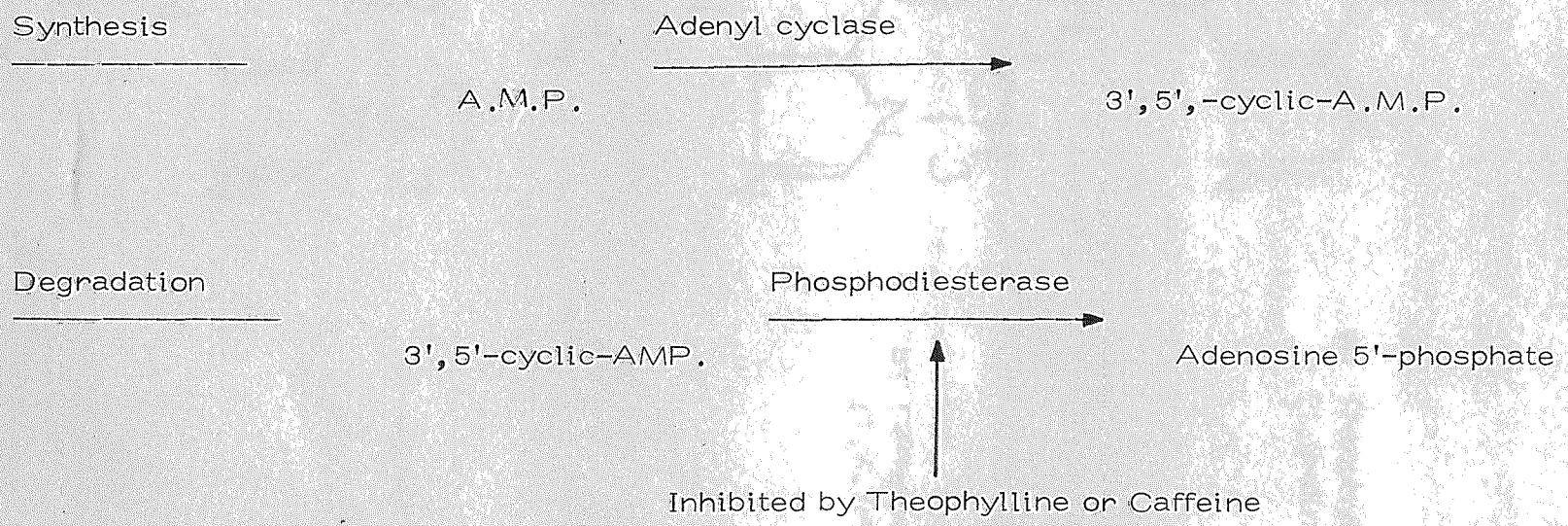
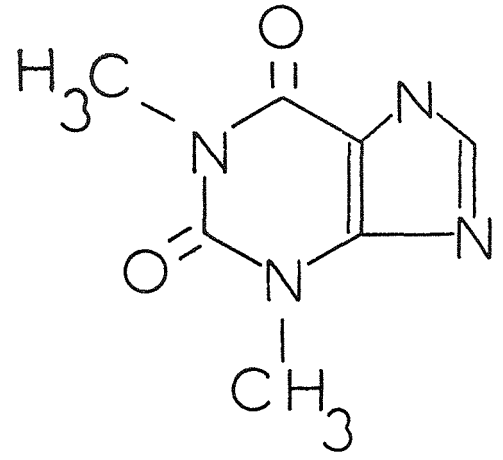
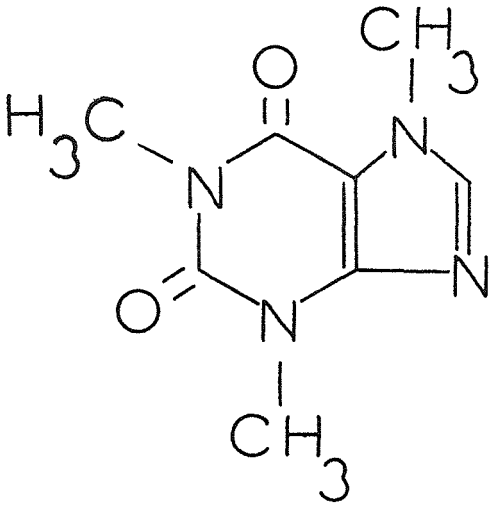


Figure 5.7.

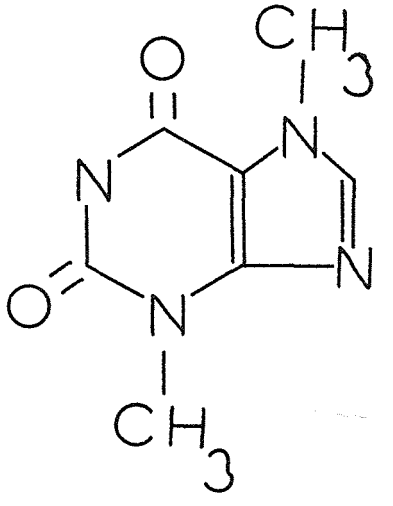
The Structure of three methylxanthines



a) Theophylline
(1,3-dimethylxanthine)



b) Caffeine
(1,3,7-trimethylxanthine)



c) Theobromine
(3,7-dimethylxanthine)

phosphodiesterase.

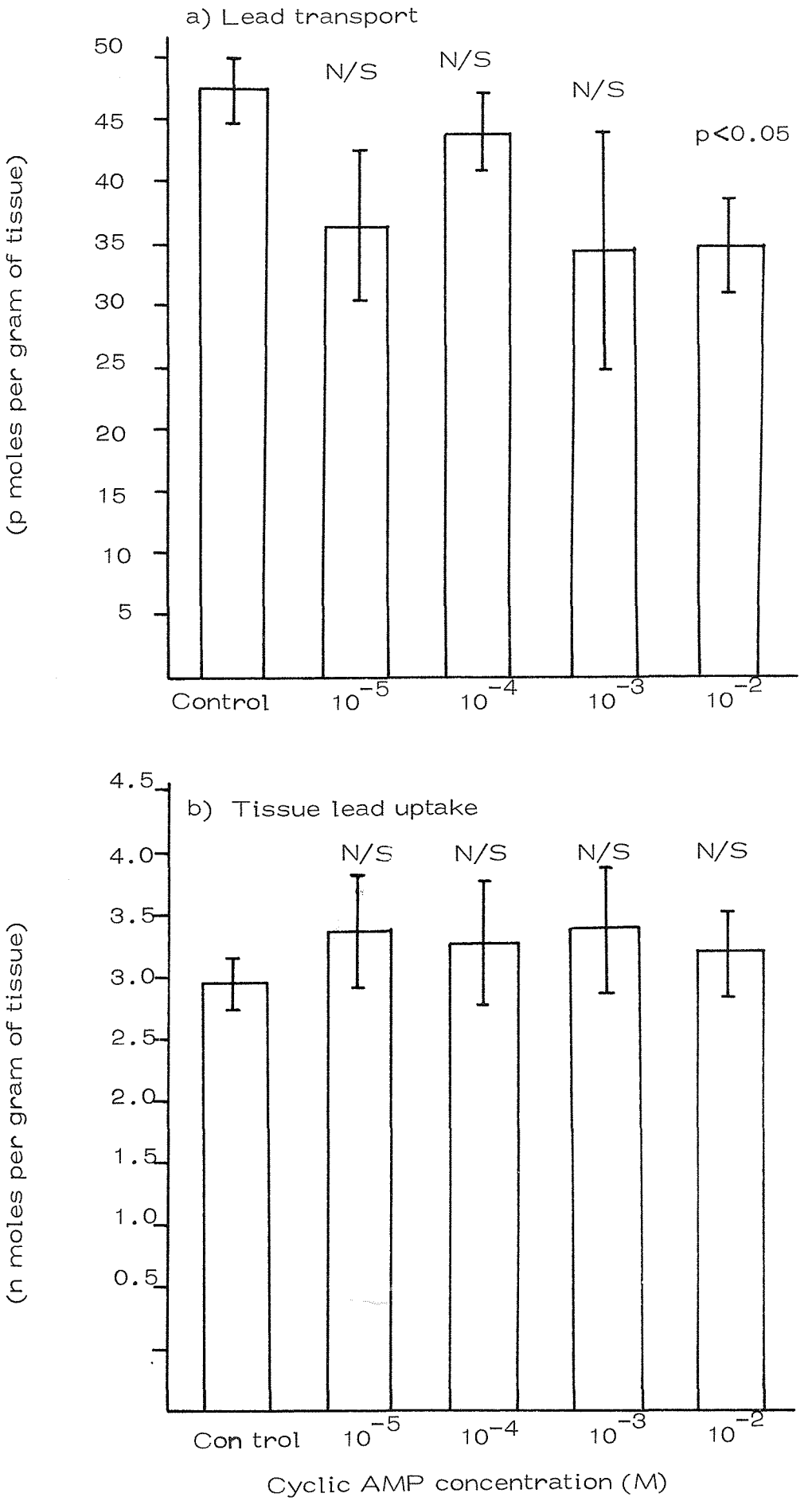
The effect of cyclic-AMP, dibutyryl-cyclic-AMP and the methylxanthines theophylline and caffeine on the uptake and transport of lead ions by rat small intestine, was investigated.

5.5. THE EFFECT OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHORIC ACID (CYCLIC-AMP) AND DIBUTYRYL-ADENOSINE 3',5'-CYCLIC MONOPHOSPHORIC ACID (db-CYCLIC-AMP) ON THE RATE OF LEAD UPTAKE AND TRANSPORT

Everted sacs were incubated for 50 minutes at $37^{o}C$ in K.H.B. buffer which contained lead acetate ($10^{-6}M$, labelled with ^{203}Pb), and various concentrations of either cyclic-AMP or db-cyclic-AMP in the mucosal media ($10^{-5}-10^{-2}M$). The incubation solutions were gassed continuously with O_2 (95%) CO_2 (5%), and the rate of lead ion transport to the serosal compartment and that of lead uptake by the intestinal tissue measured as described in Section 2.4.

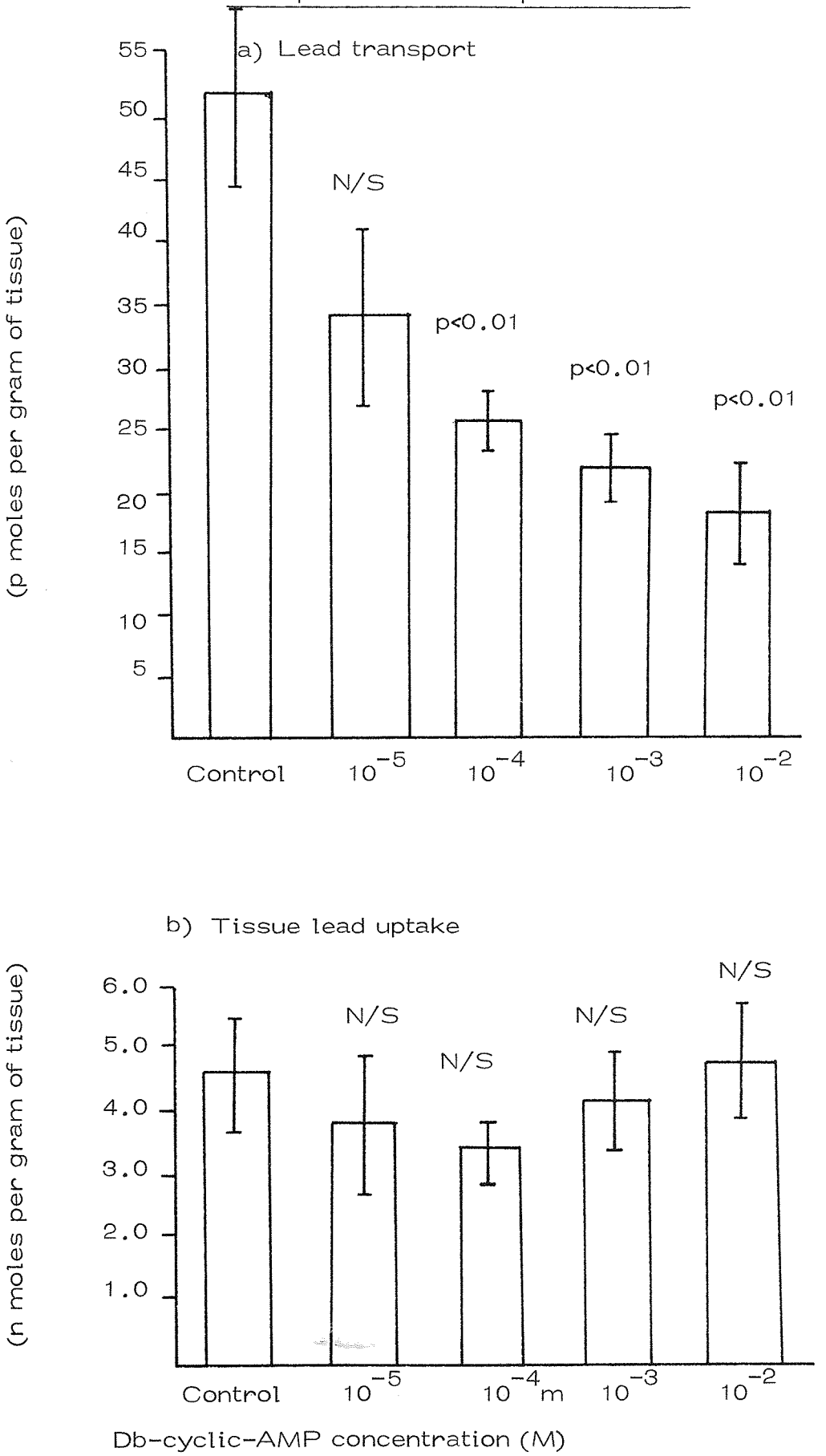
The rate of lead ion transport to the serosal compartment was decreased by the presence of both cyclic-AMP and more obviously by db-cyclic-AMP at all of the concentrations investigated, (Figures 5.8 a and 5.9a). However, the decrease of lead transport was significant in the presence of cyclic-AMP at a concentration of $10^{-2}M$ ($p < 0.05$); whereas, there was progress-

Figure 5.8. The effect of 3',5',-cyclic-AMP on the transport and tissue uptake of lead



Each point is the mean value of six experimental observations ±

Figure 5.9. The effect of dibutyryl-cyclic-AMP on the transport and tissue uptake of lead



Each point is the mean value of six experimental observations $\pm$ standard error of the mean

ive decrease in transport rate with increasing concentration of db-cyclic-AMP that became significant at 10^{-4}M db-cyclic AMP and in excess, ($p < 0.01$).

The presence of either cyclic-AMP or db-cyclic-AMP at any of the concentrations investigated, had no significant effect on the rate of lead ion uptake by intestinal tissue, (Figures 5.8b and 5.9 b).

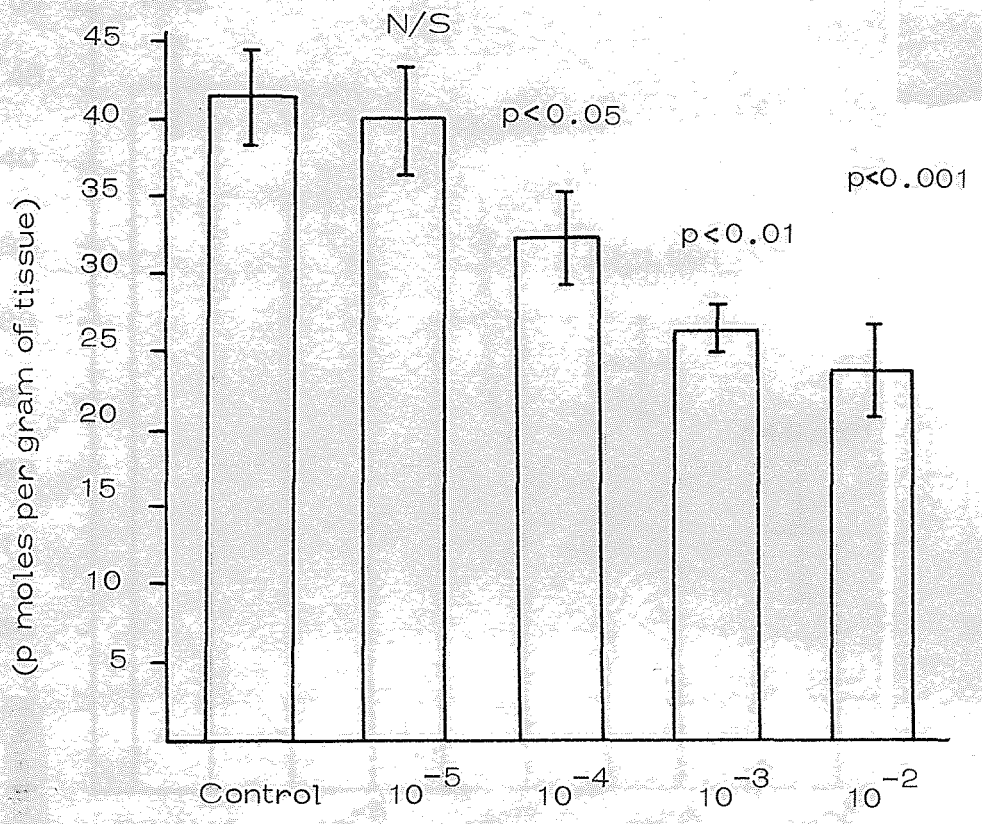
5.6. THE EFFECT OF THEOPHYLLINE, AMINOPHYLLINE AND CAFFEINE ON THE RATE OF TRANSPORT AND TISSUE UPTAKE OF LEAD

Everted sacs were incubated for 50 minutes at 37^{o}C in K.H.B. buffer which contained lead acetate, (10^{-6}M labelled with ^{203}Pb) and various concentrations (10^{-5} - 10^{-2}M) of either theophylline, aminophylline or caffeine, and gassed with O_2 (95%) CO_2 (5%). Lead ion transport to the serosal compartment, and uptake by intestinal tissue was measured as described in Section 2.4.

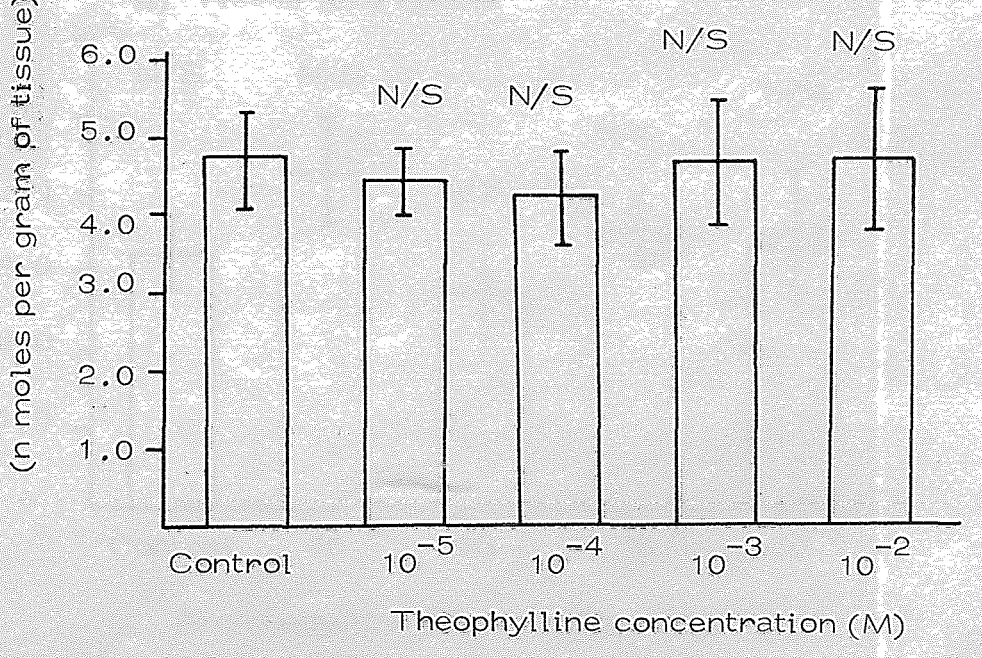
The rates of lead ion transport to the serosal compartment decreased progressively in the presence of increasing mucosal concentrations of theophylline and aminophylline, (Figures 5.10 a and 5.11 a). Caffeine however, did not produce any greater decrease in transport rate when present at greater concentrations, (Figure 5.12 a). Caffeine produced the greatest effect at the lowest concentration, with a 70% reduction at 10^{-5}M, ($p < 0.001$).

Figure 5.10 The effect of Theophylline on the transport and tissue uptake of lead

a) Lead transport



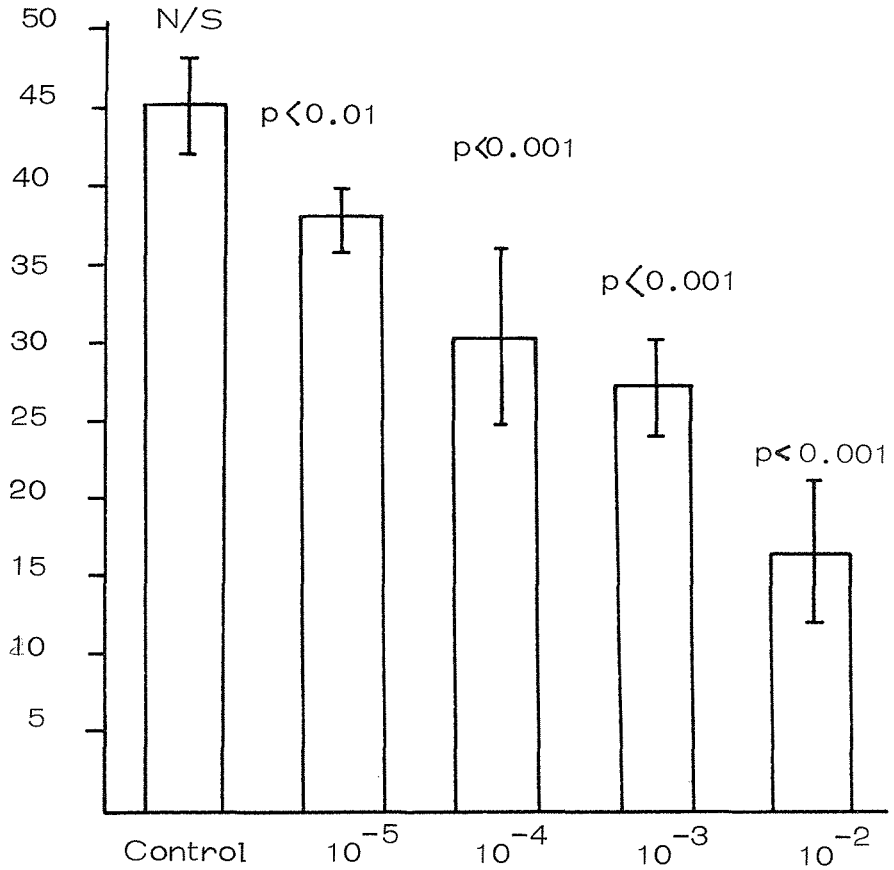
b) Tissue lead uptake



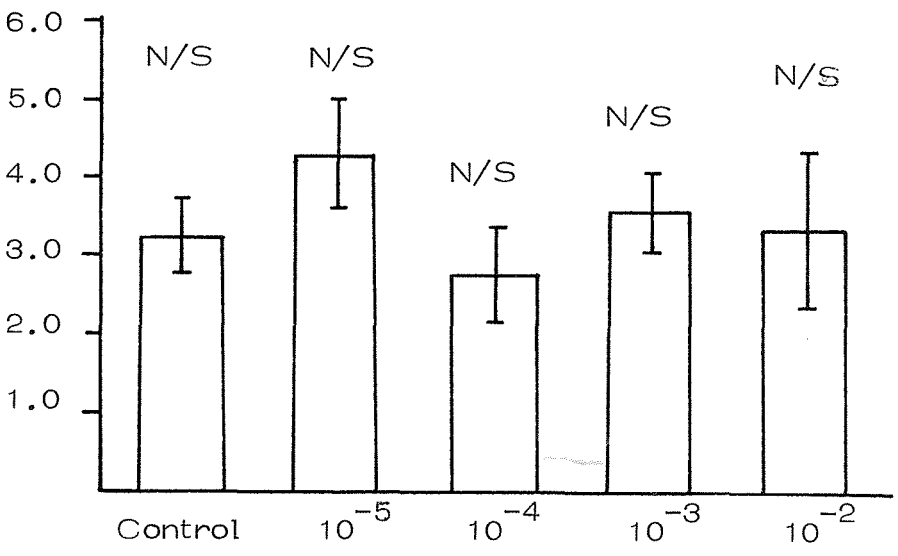
Each point is the mean value of six experimental observations ± standard

Figure 5.11 The effect of aminophylline on the transport and tissue uptake of lead

a) Lead transport



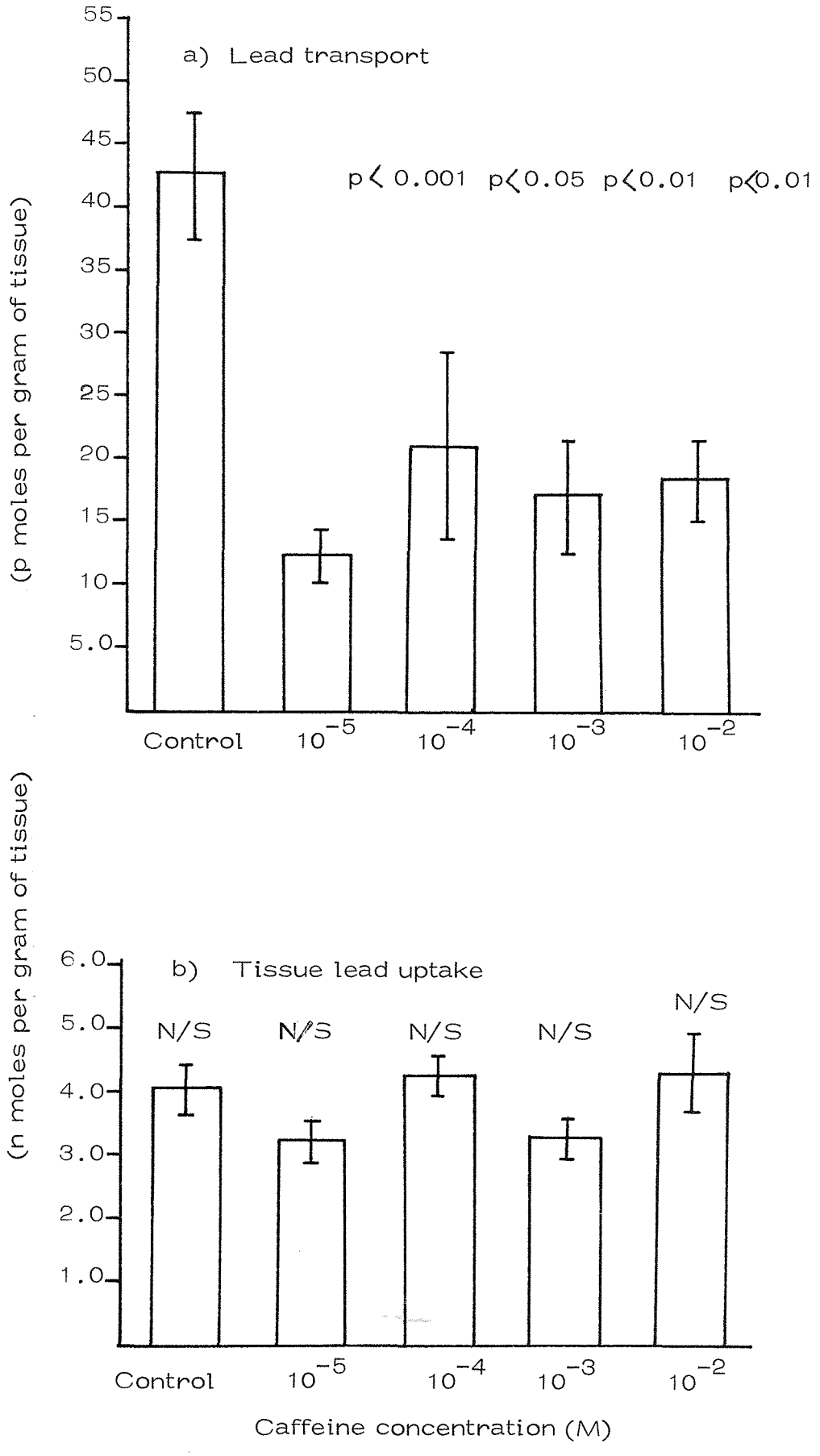
b) Tissue lead uptake



Aminophylline concentration (M)

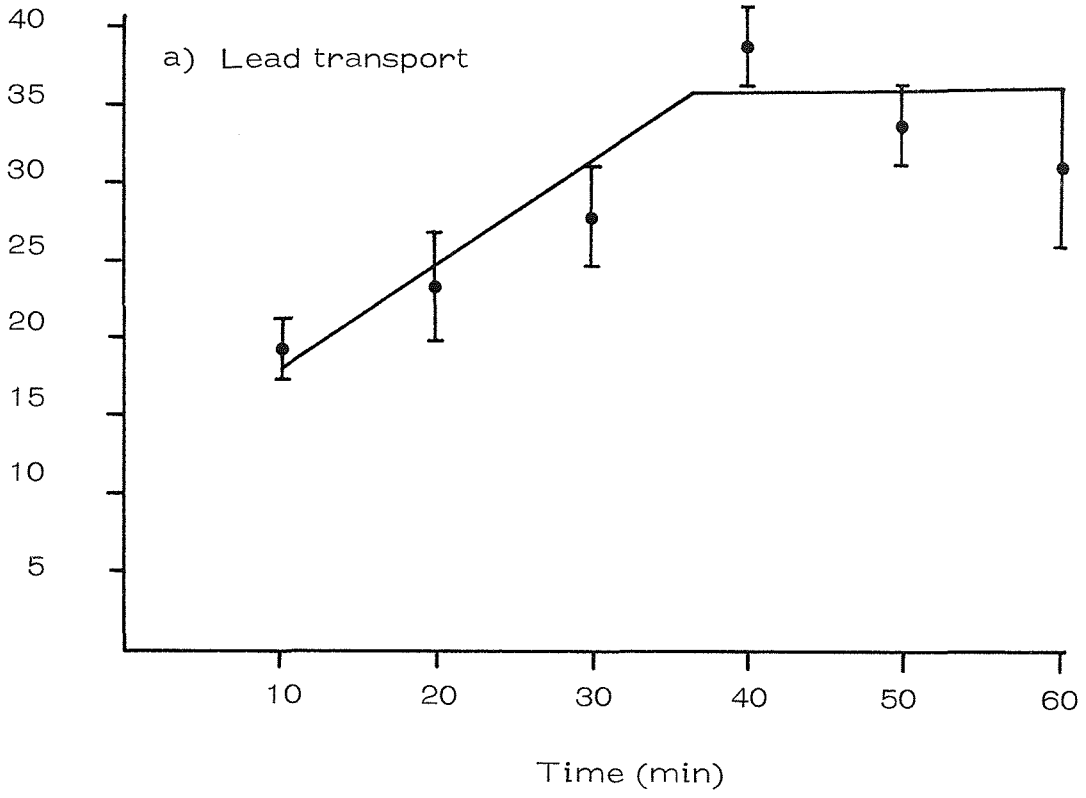
Each point is the mean value of six experimental observations ± standard error of the mean

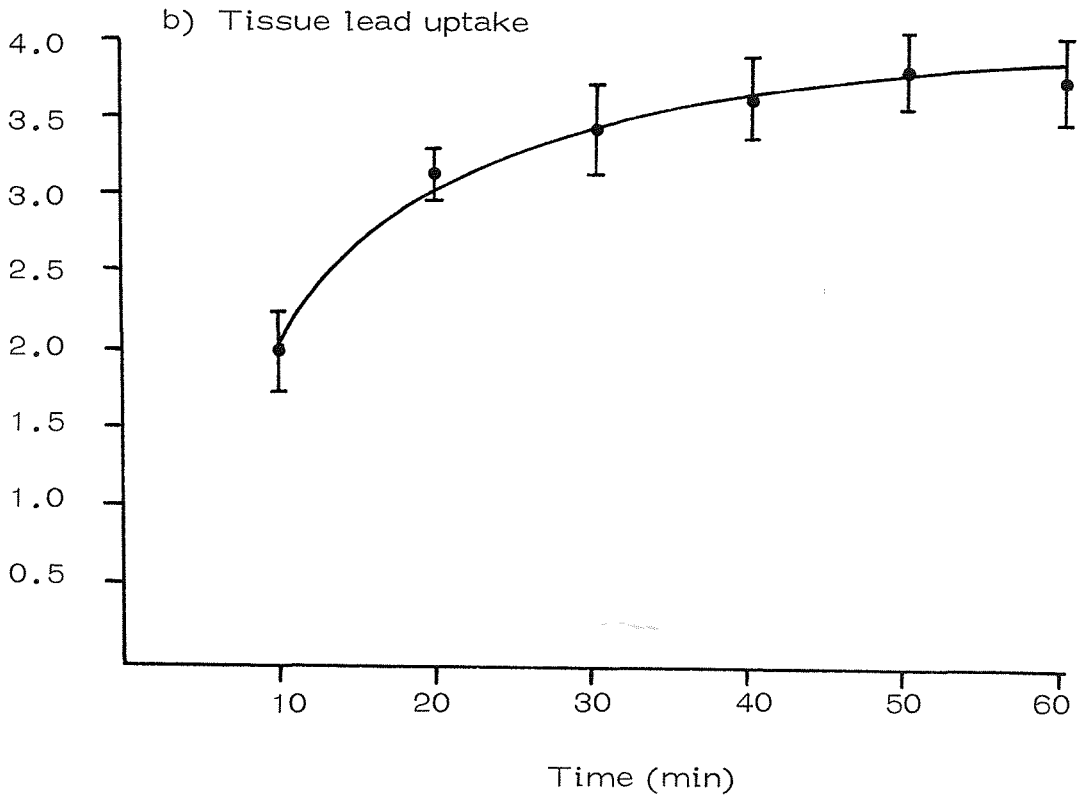
Figure 5.12 The effect of caffeine on the transport and tissue uptake of lead



Each point is the mean value of six experimental observations ± standard error of the mean

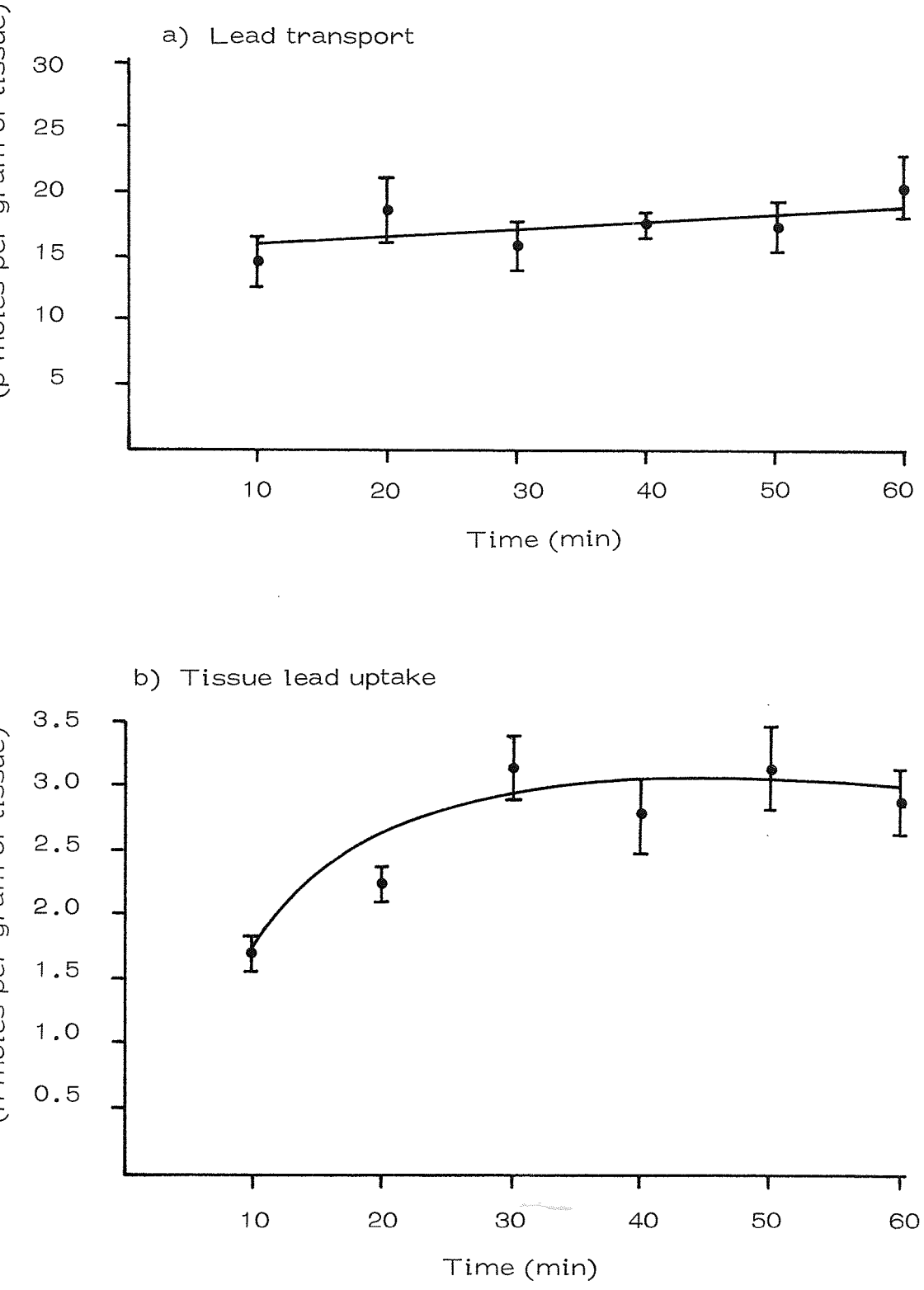
Figure 5.13 A time based investigation of the effect of Theophylline (10^{-2}M) on the rate of transport and tissue uptake of lead





Each point is the mean value of six experimental observations ± standard error of the mean

Figure 5.14 A time based investigation of the effect of Aminophylline (10^{-2}M) on the rate of transport and tissue uptake of lead



Each point is the mean value of six experimental observations $\pm$ standard error of the mean

Aminophylline was less potent than caffeine but more potent than theophylline, with a 33% reduction in lead transport when present at a mucosal concentration of 10^{-4}M, ($p<0.01$). Theophylline was least effective with a 22% reduction when present at a mucosal concentration of 10^{-4}M ($p<0.05$). The presence of either theophylline, aminophylline or caffeine at any of the concentrations investigated (10^{-5} - 10^{-2}M), did not cause any significant difference in the rate of lead ion uptake by intestinal tissue, (Figures 5.10b to 5.14 b).

Everted sacs were incubated for shorter time periods in the presence of either 10^{-2}M theophylline or aminophylline. It was observed that the transport of lead ions to the serosal compartment ceased after 35 minutes of incubation in the presence of theophylline, (Figure 5.13 a) and after 10 minutes in the presence of aminophylline, (Figure 5.14 a).

5.7. THE EFFECT OF THEOPHYLLINE (10^{-2}M) AND AMINOPHYLLINE (10^{-2}M) ON THE TRANSMURAL POTENTIAL DIFFERENCE ACROSS THE TISSUE

Cannulated everted sacs prepared from the mid-jejunum were submerged in K.H.B. buffer that contained either theophylline (10^{-2}M) or aminophylline (10^{-2}M) on the mucosal side only, and continuously gassed with O_2 (95%) CO_2 (5%). Salt bridges were attached and the transmural potential difference measured at 10 minute intervals over a 60 minute incubation period. Full experimental details are

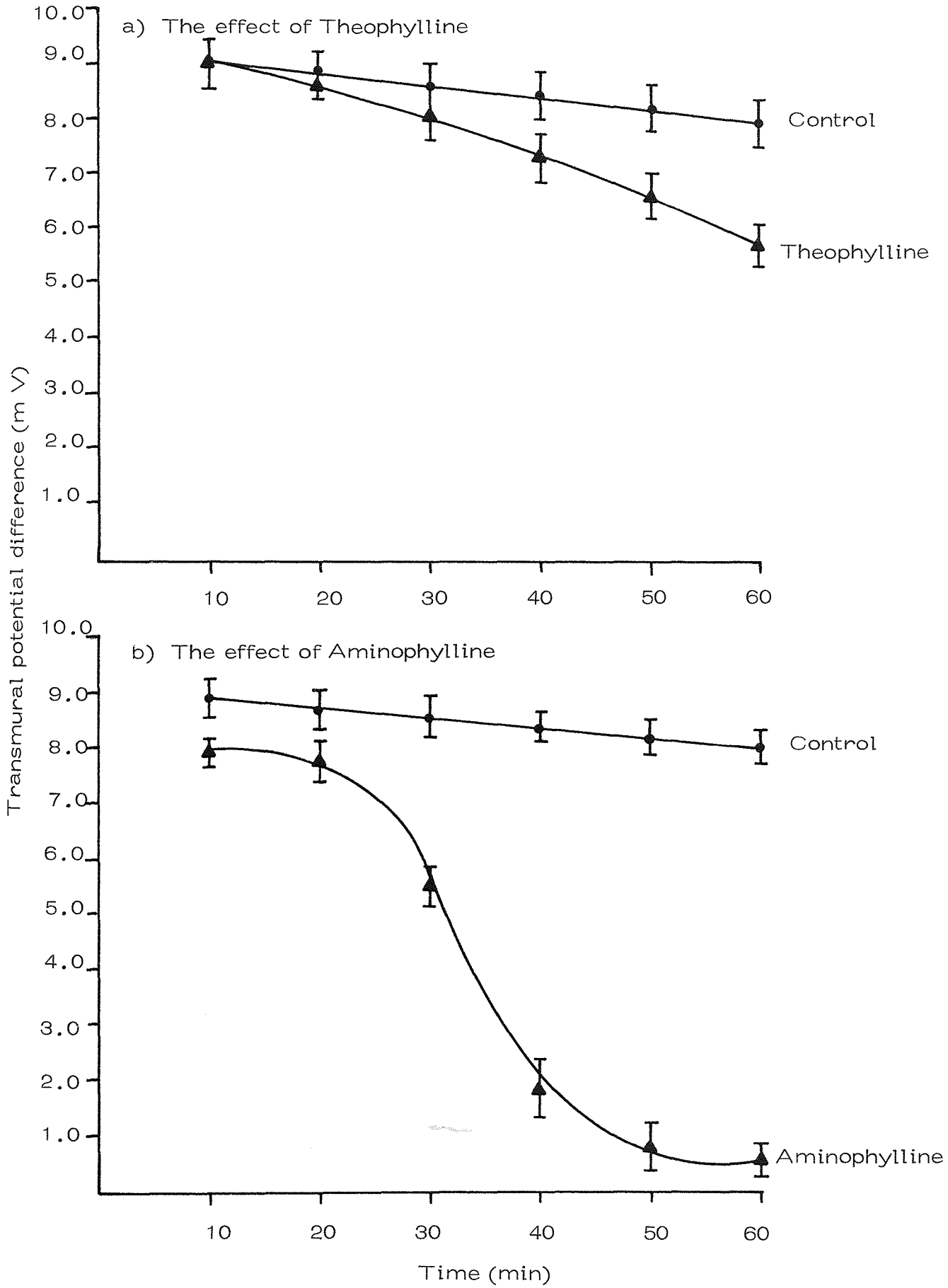
given in Section 2.6.

The presence of either theophylline (10^{-2}M) or aminophylline (10^{-2}M) caused a decrease in the transmural potential difference with time. The decrease became significantly different from a control value ($p < 0.05$) after 30 minutes of incubation in the presence of theophylline, (Figure 5.15a), and after only 10 minutes of incubation in the presence of aminophylline, (Figure 5.15 b).

5.8. DISCUSSION

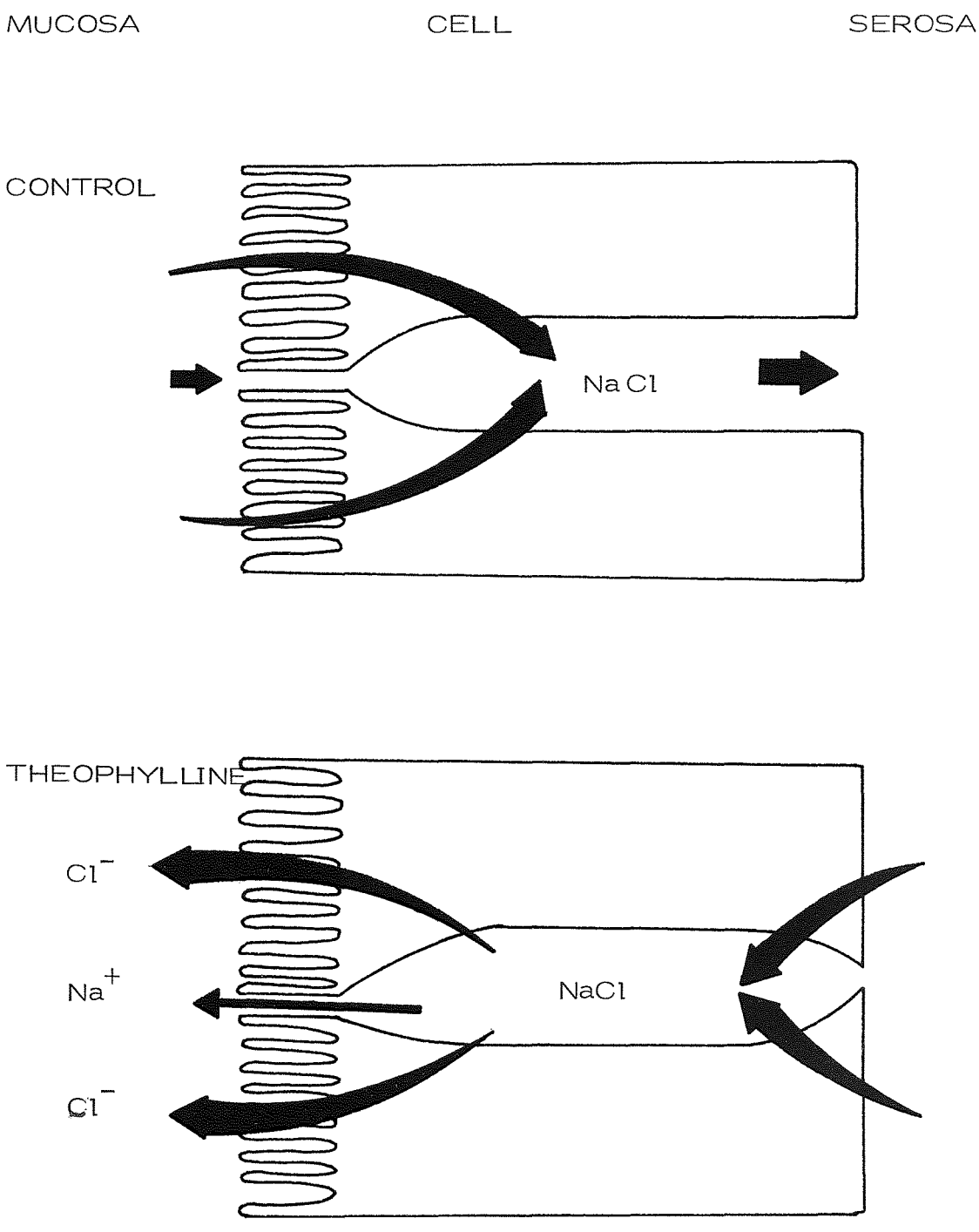
The presence of theophylline in the intestinal lumen has been reported to evoke electrolyte secretion and reduce mucosal conductance, (Powell, 1974). It is believed that theophylline inhibits phosphodiesterase activity and consequently increases the intracellular concentration of cyclic-AMP., (Beubler and Lembeck, 1980). The mechanism involved in electrolyete secretion is as follows :- elevated concentrations of intracellular cyclic-AMP, initiate increased apical and basolateral membrane permeability to chloride ions. Chloride ions consequently 'leak' out of the hypertonic intercellular spaces into the intestinal lumen, and sodium ions, which are electrogenically coupled to chloride ions, move into the intestinal lumen through the tight junctions, hence sodium and chloride ions are secreted, (Figure 5.16., Naftalin and Simmons, 1979, and Ilundain and Naftalin, 1979). When fluid is induced to flow from the serosa to

Figure 5.15 The effect of Theophylline (10^{-2}M) and Aminophylline (10^{-2}M) upon the transmural potential difference across the tissue preparation



Each point is the mean value of six experimental observations ± standard error of the mean

Figure 5.16 The proposed model for Theophylline induced electrolyte secretion (Naftalin and Simmons, 1979)



the mucosa in response to electrolyte movement, a collapse of the intercellular spaces occurs which reduces transepithelial conductance, (Smulders et al., 1972). This scheme of events is further confirmed by other authors, (Corbett et al., 1977 and Turnberg, 1978).

The experimental data demonstrate that the rate of lead ion transport is dependent upon the extracellular cyclic-AMP concentration and therefore may be dependent upon the intracellular cyclic-AMP level, (Figure 5.8a). The normal level of intracellular cyclic-AMP is approximately 10^{-7}M (Robison, et al., 1971) and an increase above this level may be expected to result in cell swelling and reduced epithelial conductance. However, a significant decrease in lead transport was observed only at an extracellular cyclic-AMP concentration of 10^{-2}M. The more lipid soluble moiety db-cyclic-AMP produced a significant decrease in the rate of lead transport when present at an extracellular concentration of 10^{-4}M, and suggests that either 1) mucosal cell membrane permeability to cyclic-AMP is low, or 2) that db-cyclic-AMP is more resistant than cyclic-AMP to break down by phosphodiesterase.

Other means of increasing the intracellular concentration of cyclic-AMP are to reduce its rate of degradation by phosphodiesterase inhibition, (Figure 5.6). The methylxanthines are known to inhibit phosphodiesterase and therefore increase the concentration of intracellular cyclic-AMP. It has been demonstrated that the presence

of the methylxan thines theophylline and caffeine can significantly decrease the rate of lead ion transport, (Figures 5.6 a to 5.10 a).

If lead ions adopt a paracellular route of transport via the tight junctions and lateral intercellular spaces, then a collapse of the intercellular spaces would be expected to lead to a progressive decrease in the rate of lead ion transport. A point would be reached where the intercellular spaces were smaller than the diameter of the lead ion, which would result in a ceasation of lead ion transport. This is indeed what was observed in the presence of theophylline (10^{-2}M, Figure 5.13 a).

The presence of aminophylline at an extracellular concentration of 10^{-2}M produced a similar effect on lead transport to theophylline, except the ceasation of lead transport occurred sooner, (Figure 5.14 a). Aminophylline is a complex of ethylenediamine and theophylline and the complex may be rendered more lipid soluble than theophylline alone.

Theophylline produces a progressively greater inhibition of lead transport when present in increasing concentrations. Caffeine, however, appears to produce its greatest effect when present at 10^{-5}M and exhibits no greater effect at higher concentrations. This can be explained by the fact that theophylline inhibits phosphodiesterase specifically, i.e., it competes with cyclic-AMP for the active site and therefore its inhibition will be concentration dependent. Caffeine, however, inhibits phosphodiesterase in a non-specific manner, i.e., it

binds to the enzyme at a site other than the active site, and induces conformational alterations that reduces the efficiency of the enzyme, (Robison et al., 1971).

Further evidence of a reduction in the extracellular route of ion transport is given by Figures 5.15a and 5.15b. Since the transmural potential difference is a measure of the steady state charge difference across the intestinal epithelium, which is itself due to a net electrolyte movement; a decrease in the overall electrolyte flux will be reflected by a decreased potential difference. This was observed in the presence of theophylline (10^{-2}M) and aminophylline (10^{-2}M).

Whatever the precise mechanism involved in lateral intercellular space collapse, its effect upon lead ion transport across the intestinal epithelium is profound. The data presented in this chapter demonstrate two major effects:

1) incubation in the presence of extracellular cyclic-AMP, or db-cyclic-AMP can significantly reduce the rate of lead ion transport; and

2) theophylline, aminophylline or caffeine can be used to mimic the same response as cyclic-AMP. The data supports a model which postulates that lead ions traverse the intestinal epithelium as a consequence of passive movement via the paracellular route, (i.e. via the tight junctions and lateral intercellular channels).

CHAPTER SIX

THE EFFECT OF DIETARY METALS ON THE TRANSPORT AND TISSUE UPTAKE OF LEAD

6.1. THE RELATIVE AMOUNTS OF METALS PRESENT IN THE DIET

The amount of metals present in food varies from relatively large amounts for essential metals, to only trace amounts for non-essential metals. Typical daily intake values for metals present in food are, zinc approximately 13 mg, iron approximately 10-15 mg, aluminium approximately 0.06-0.97 mg, cadmium approximately 0.01-0.03 mg, and mercury approximately 0.005-0.02 mg, (Monier-Williams, 1949 and D.H.S.S., 1980). Lead intake has been estimated to be approximately 0.1 - 0.3 mg per day (Pickford, 1981 and D.H.S.S., 1980).

6.2. THE INFLUENCE OF OTHER METALS ON LEAD TRANSPORT

Several authors have reported observations in both rats and humans concerning the effects of dietary metals on the absorption of lead. Variations from physiological quantities of dietary calcium has been demonstrated to significantly affect lead absorption, (Barton et al., 1978). High does of dietary lead have been demonstrated to significantly decrease the passive transfer of calcium and strontium in rats, (Gruden et al., 1974). It has been suggested that lead, calcium and strontium traverse the intestinal epithelium via the same route and that lead may cause an obstruction of the route which reduces transport efficiency. A common pathway for the absorption of calcium and other minerals has been postulated to account for the competition

observed between divalent metals, (Meredith et al, 1977).

The presence of excess zinc, like calcium, has been reported to decrease lead absorption, but the data are explained by competition for a binding protein present in the intestinal mucosa, (Cerklewski and Forbes, 1976). Further, absorption has been reported to be increased in animals that are in an iron deficient state, (Six and Goyer, 1972). The data may also be explained as competition between lead and iron for sites on intestinal iron binding proteins.

Although the precise mechanism which initiates the effect of dietary metals on lead absorption is not clearly understood, the literature presents two possible mechanisms. The first is a common pathway, for all metal ions, where the relative concentration of each metal in the gut lumen governs the extent of absorption of the metal. The second mechanism involves the participation of mucosal binding proteins that are present for the active absorption of essential metals such as iron and calcium. Under conditions of iron or calcium deficiency, the binding proteins are utilised by other divalent metals. The data presented in Chapter Four of this thesis are inconsistent with the concept of protein mediated lead absorption, therefore, no attempt was made to investigate binding proteins further. However, the former mechanism of a common pathway was investigated for a variety of metals.

The data is presented in two parts. The first part deals with

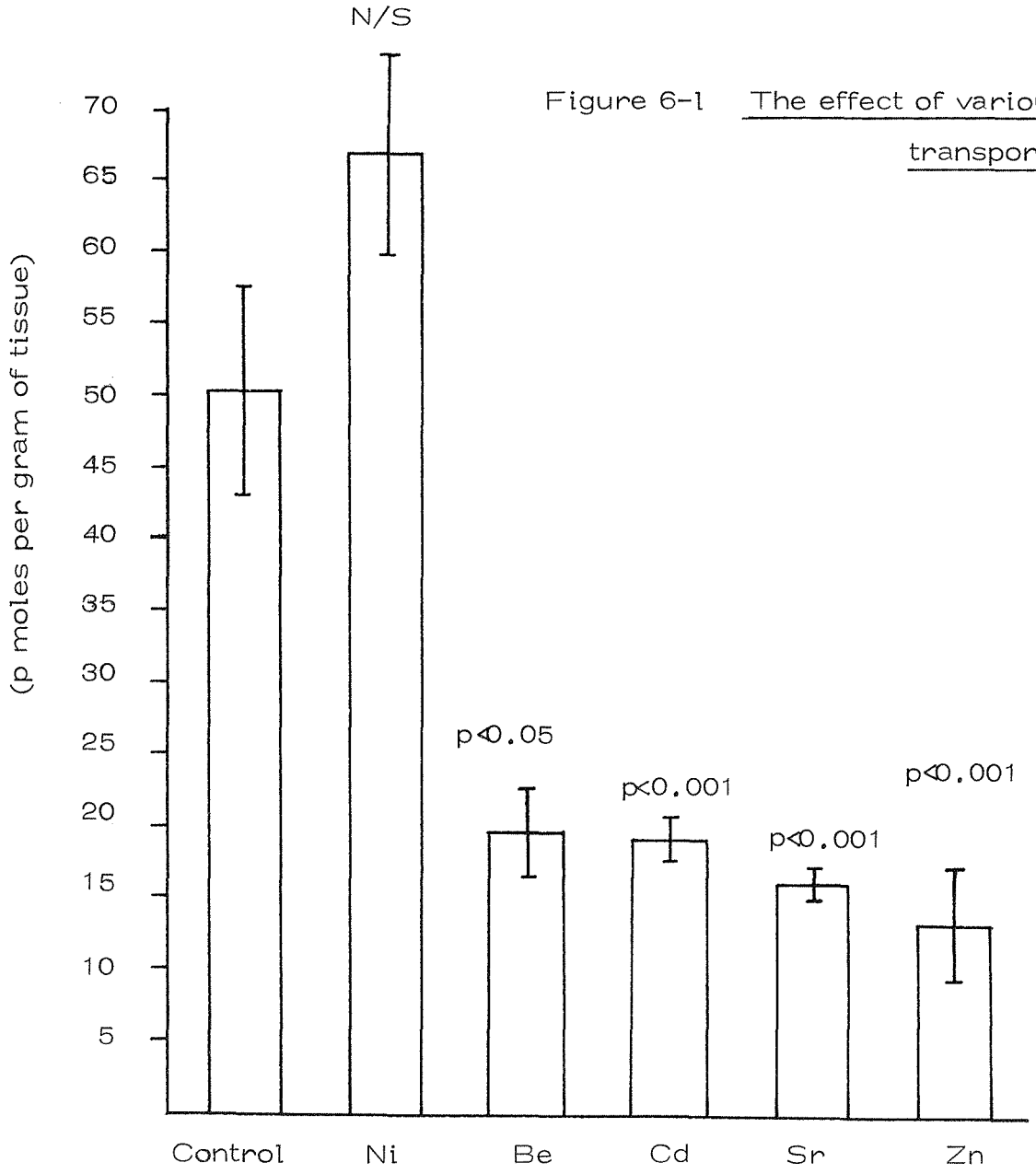
ten metals that are present in the normal diet in trace amounts and their effect on the rate of lead transport. The second part deals with five metals that demonstrated an interesting effect on the uptake of lead by intestinal tissue which may be important in the overall process of lead transport.

6.3. THE EFFECT OF DIVALENT AND TRIVALENT METAL CATIONS ON THE RATE OF LEAD TRANSPORT

Everted sacs were incubated at 37°C for 50 minutes in Tris buffer that contained various divalent metals (Ni, Be, Cd, Sr or Zn) or trivalent metals (Cr, Al, Fe, Ce or La) at a concentration of 10^{-2}M and with 10^{-6}M lead acetate labelled with ^{203}Pb, and gassed continuously with O_2 (100%). Lead transport was measured as described in Section 2-4.

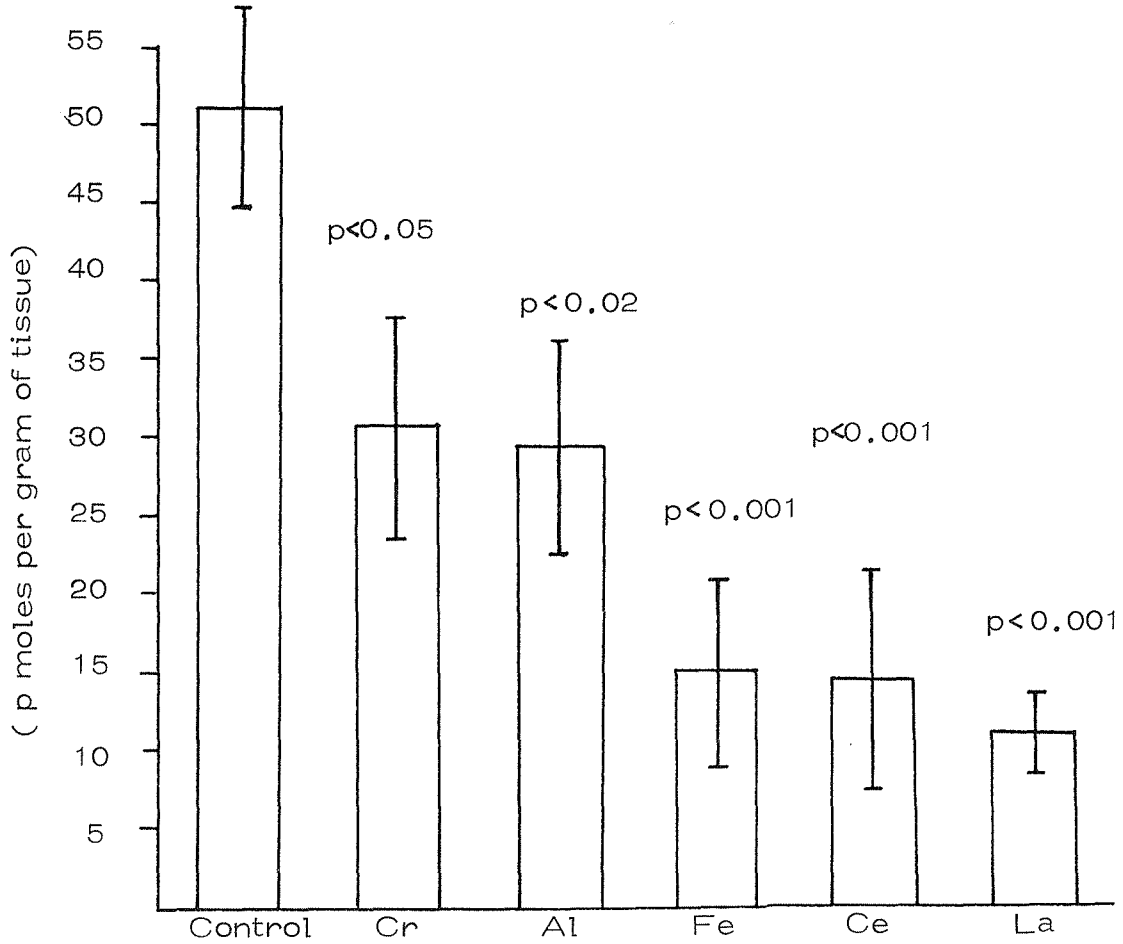
The rate of lead transport was significantly reduced in the presence of 10^{-2}M beryllium ($p < 0.05$), cadmium ($p < 0.001$), strontium ($p < 0.001$) and zinc ions ($p < 0.001$). The presence of nickel ions at 10^{-2}M however, produced no significant effect on the rate of lead transport, (Figure 6.1). The presence of each of the trivalent metals produced a significant reduction in lead transport, (Figure 6.2). The order of potency was lanthanium ($p < 0.001$) cerium ($p < 0.001$) iron ($p < 0.001$) aluminium ($p < 0.02$) chromium ($p < 0.05$).

Figure 6-1 The effect of various divalent metal cations on the transport of lead



Each value is the mean of six experimental observations ± standard error of the mean

Figure 6.2. The effect of various trivalent metal cations on lead transport



Each value is the mean of six experimental observations ± standard error of the mean

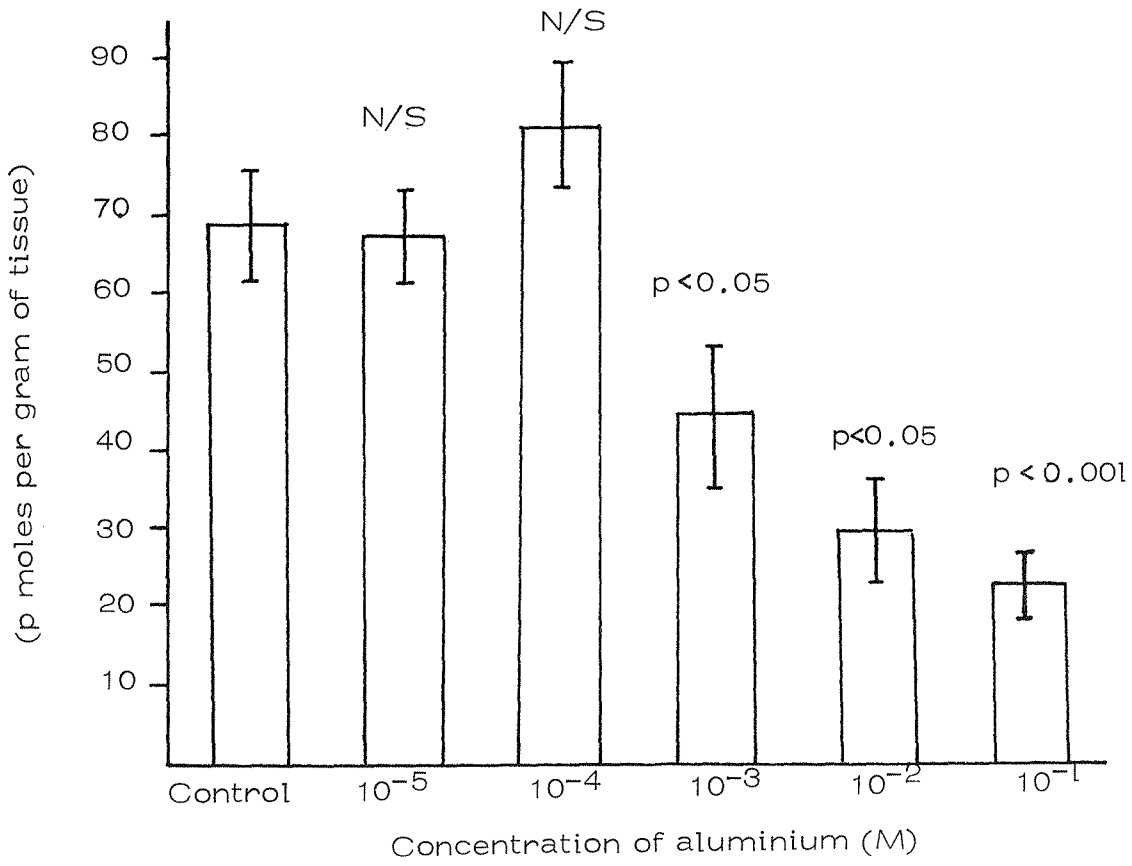
The effect of zinc, iron and aluminium on lead transport was investigated in more detail, by incubating everted sacs as before with 10^{-6}M lead acetate in the presence of various concentrations of the second metal cation, (10^{-6}M to 10^{-1}M). The rate of lead transport was significantly reduced in the presence of excess aluminium ($p < 0.05$ at 10^{-3}M, Figure 6.3), and iron ($p < 0.05$ at 10^{-4}M, Figure 6.4), and at equimolar concentration of zinc ($p < 0.01$ at 10^{-6}M, Figure 6.5). Higher concentrations of each metal produced a progressive decrease in the rate of lead transport.

6.4. DISCUSSION

Few studies concerned with passive ion movements has been conducted on the small intestine, most fundamental electrophysiological measurements have been obtained from investigations carried out on rabbit gallbladder. Barry et al., (1971) and Wright and Diamond (1968) report that the transepithelial membrane resistance of gallbladder, as determined by microelectrode measurements, is much lower than that obtained across a single epithelial cell membrane, indicating a paracellular route for passive ion permeation. Further, the main barrier to ion permeation was more consistent with a membrane considerably thicker than that presented by the epithelial cell membrane.

The tight junctions are regions of cell to cell contact, with contact lengths of 200-300 nm, compared to cell membranes which are

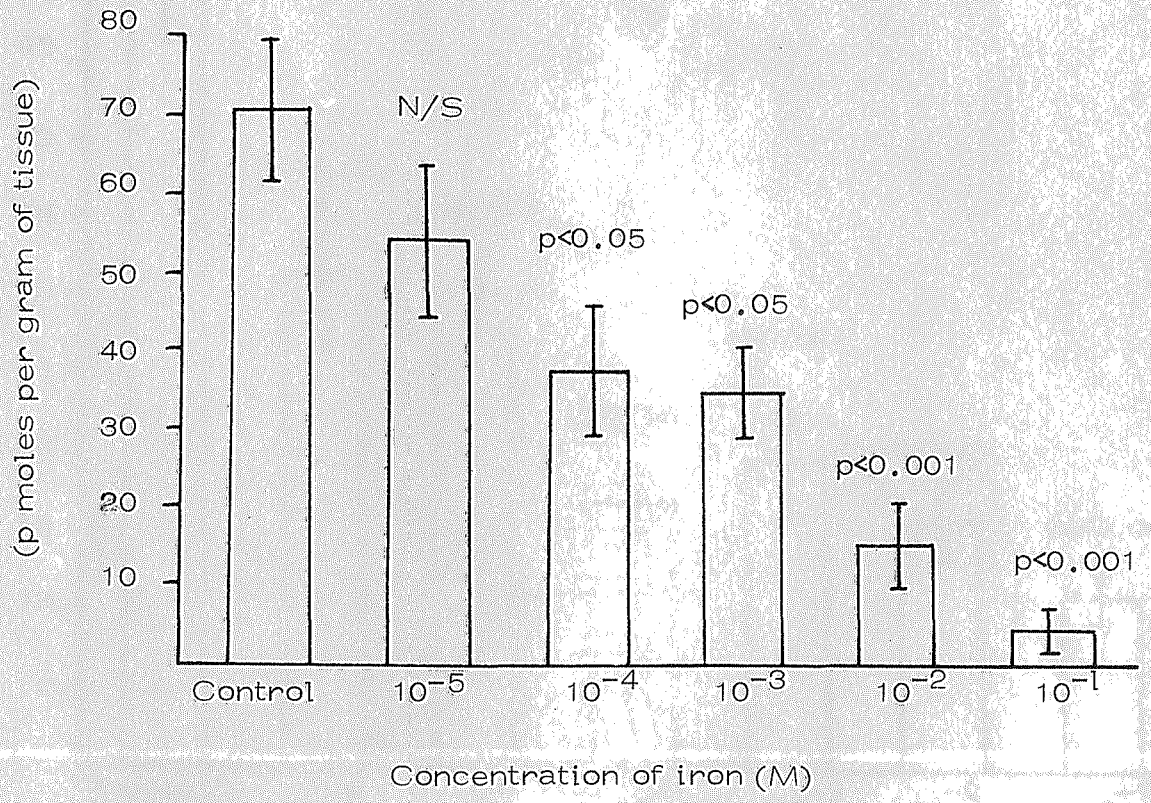
Figure 6.3 The effect of aluminium on lead transport



Each value is the mean of six experimental observations ± standard error the mean

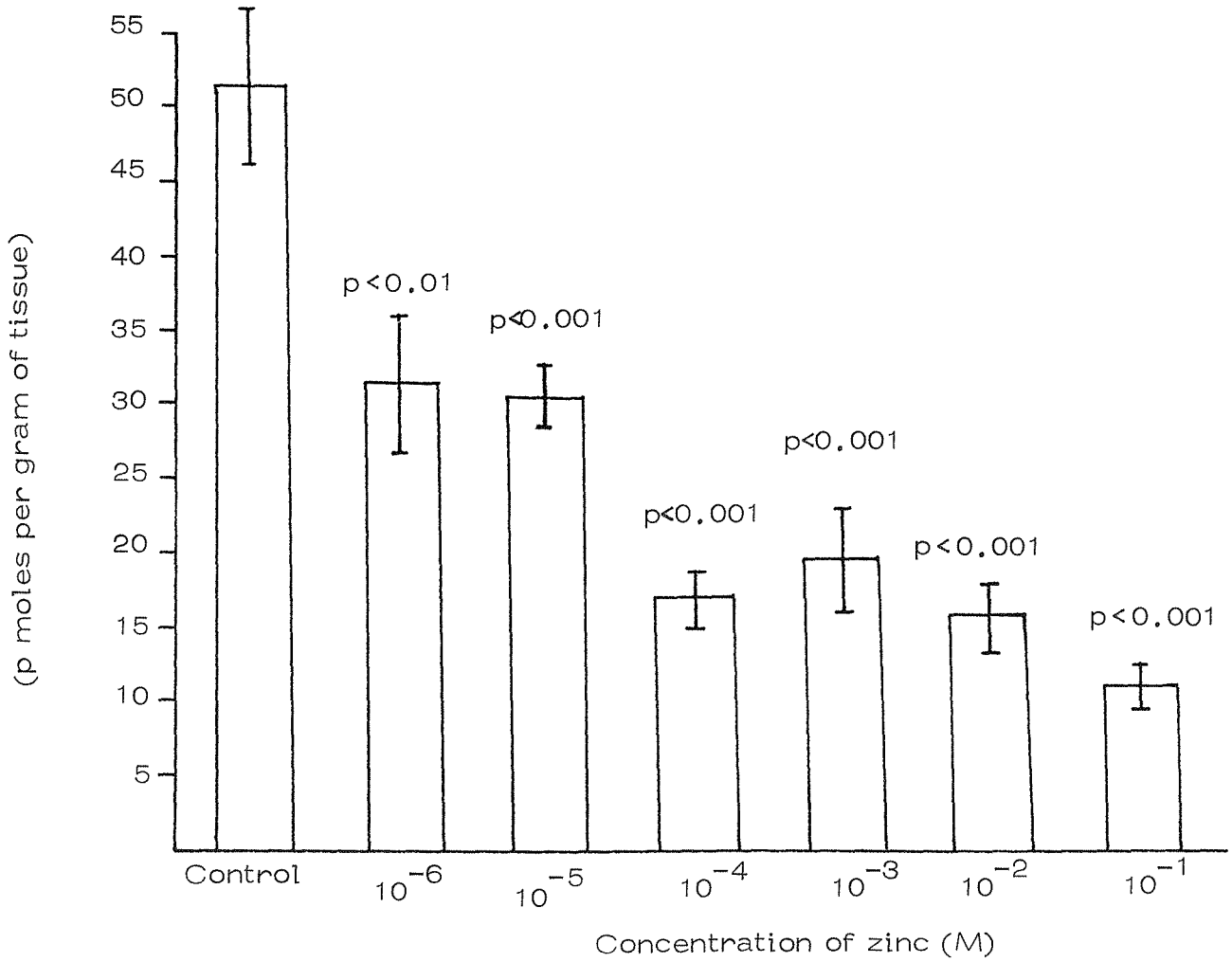
Figure 6.4.

The effect of iron on the transport of lead



Each value is the mean of six experimental observations ± standard error of the mean

Figure 6.5. The effect of zinc on lead transport



Each value is the mean of six experimental observations ± standard error of the mean

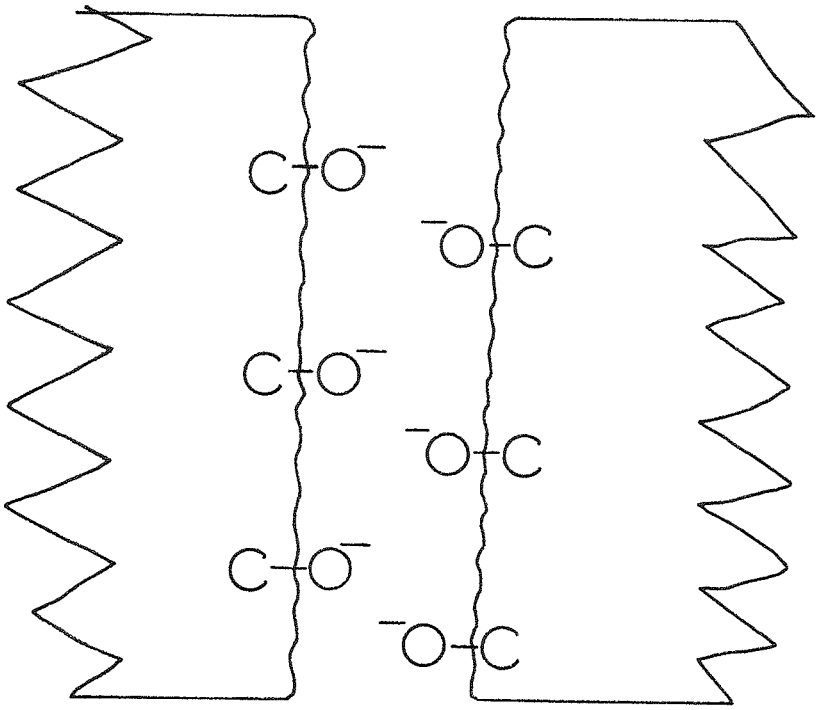
approximately 8 nm thick. Thus the tight junctions were considered to be the most probable barrier to transepithelial permeation. The gallbladder also demonstrates symmetrical permeability properties typical of single membranes, in contrast to differential permeability exhibited by the apical and basolateral membranes of epithelial cells. The permeability of the gallbladder epithelium to different ions exhibited rates of transport that were not greatly different from each other, and were indicative of passive ion transport via an aqueous route, rather than a route through lipid.

Rabbit intestine has been demonstrated to have linear conductance/concentration and current/voltage relations characteristic of leaky epithelia. It is therefore likely that the intestine is similar to the gallbladder with respect to the mode of passive ion permeation. As most of the passive ion flux across the intestinal epithelium occurs via a paracellular route, then the site at which competition occurs must reside at the tight junctions.

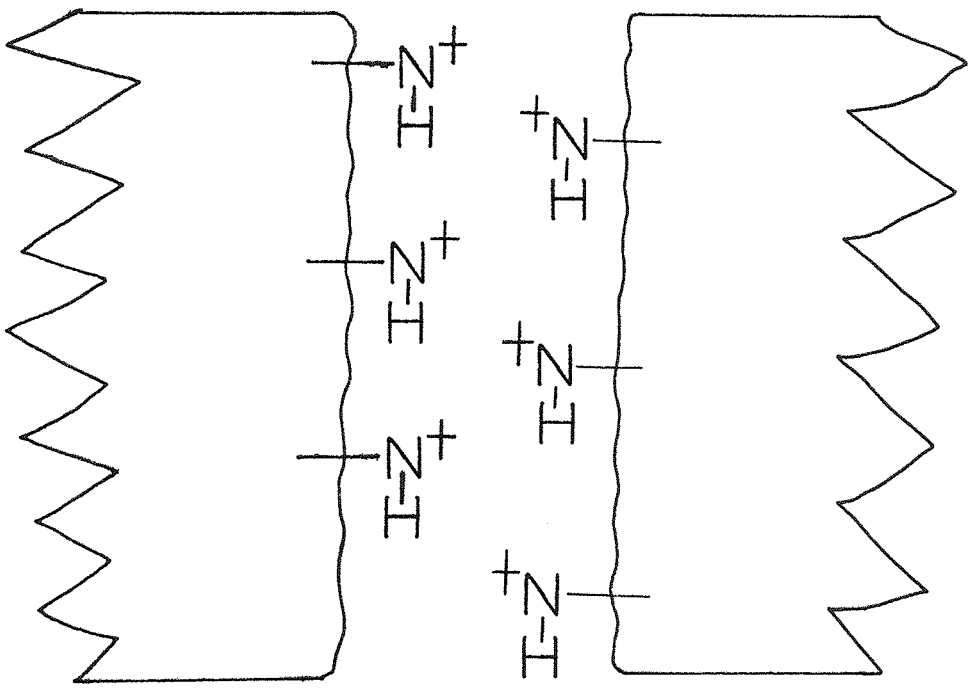
It is believed that a process of ion-exchange occurs within the tight junction complexes, between fixed membrane charged groups and counter-ions, (Frizzell and Schultz, 1972, Figure 6.6). In gallbladder tight junctions the chemical identity of the fixed charged groups has not been determined unequivocally, but it has been estimated that acidic groups with pKa values in the pH range 2-4 are involved. Acidic groups that are compatible with pKa values in the pH range 2-4

Figure 6.6. The structural basis of cation and anion selectivity in membranes

a) Cation selective channel



b) Anion selective channel



are either carboxyl or phosphoric acid residues. Basic groups known to be present within the tight junction complexes have similar pKa values to the acidic groups, and are probably nitrogen functions.

Figures 6.1 and 6.2 demonstrate a competition between lead and other metal ions for transport via the paracellular route. The sequential order of effect obtained is;

$$La^{3+} > Zn^{2+} > Ce^{3+} > Fe^{3+} > Sr^{2+} > Cd^{2+} > Be^{2+} > Al^{3+} > Cr^{3+} > Ni^{2+}$$

The metal ions may be grouped together into four distinct groups with respect to their effect on lead transport. Group I consists of the nickel ion which produced no significant reduction in lead transport. Group III comprises of chromium and aluminium which produced an approximate 40% reduction in lead transport. Group II comprises of beryllium, cadmium, strontium and zinc, and group IV iron, cerium and lanthanium, respectively, which produced approximately a 70% reduction in lead transport. The factors that determine the extent of competition for transport via a paracellular route will now be considered.

The effect of ionic size

The simplest model that explains passive ion movement is one whereby the tight junction complexes form channels in which only the channel size hinders passive ion permeation. The rate of ion transport will therefore be determined by 1) the size of the channel,

2) the relative concentrations of ions, and 3) the relative sizes of the ions. During the present experimentation the channel size and ionic concentrations were kept constant, so that the effect of the ionic size of the competing metals could be investigated. Table 6.1 displays various radii of the metals used in this investigation. All ionic radii of the trivalent metals are smaller than that of lead, and the only trend of effect based on ionic size that can be interpreted from the experimental data is that lanthanium and strontium have a similar ionic radius to lead and produce a marked effect on lead transport. Chromium and aluminium are approximately half the size of lead and produce a smaller reduction of lead transport than lanthanium or strontium. However, no trend exists to explain why zinc and iron produce a similar effect to lanthanium when their ionic radii are approximately half the size of lanthanium, or similarly why chromium has a smaller effect than iron when their ionic radii are of a similar size. Beryllium too is anomalous, since it produces a great reduction of lead transport and is only approximately one quarter of the size of lead, and nickel which is approximately half the size of lead has no effect at all on lead transport.

The effect of hydrated size

The metal ions investigated would to some extent form aquo-ions in aqueous solution, and so the species involved would be larger than the ionic radius. Generally, the smaller the cation, the

TABLE 6.1. VALUES OF VARIOUS RADII OF METAL CATIONS

(a) TRIVALENT METAL CATIONS

FUNCTION (Å)	La	Fe	Al	Cr	Pb
IONIC RADIUS	1.15	0,64	0.50	0.69	1.20
COVALENT RADIUS	1.69	1.17	1.25	1.18	1.54
METALLIC RADIUS	1.88	1.26	1.43	1.30	1.75
APPROXIMATE HYDRATED RADIUS	9.00	9.00	9.00	9.00	4.50

(b) DIVALENT METAL CATIONS

FUNCTION (Å)	Zn	Sr	Cd	Be	Ni	Pb
IONIC RADIUS	0.74	1.13	0.97	0.31	0.62	1.20
COVALENT RADIUS	1.25	1.91	1.44	0.89	1.15	1.54
METALLIC RADIUS	1.37	2.15	1.54	1.12	1.25	1.75
APPROXIMATE HYDRATED RADIUS	6.00	5.00	5.00	8.00	6.00	4.50

greater will be the interaction with water. The extent of hydration of an ion is not limited to any specific extent, since several layers of water molecules can be associated with the ion, but with decreasing strength of association as the number of water layers increase. The net result is that the cation with the smallest ionic radius would have the largest hydrated radius. The exact size of hydrated ions is difficult to measure but approximate values are given in Tables 6.1 a and 6.1 b. The trivalent metal species have approximately the same hydrated sizes and are all larger than lead, however, their effects on lead transport are different The difference, for example, between lanthanium and aluminium cannot be explained on the basis of hydrated size. Similarly with the divalent metal species, the large reduction in lead transport demonstrated by zinc cannot be accounted for by hydrated ion size alone, since nickel which has a similar hydrated size has no effect on lead transport. It is clear that the hydration sizes of different metal ions considered per se does not explain the relative differences in lead transport observed.

Thermodynamic considerations

It has been postulated (Diamond and Wright, 1969) that biological membrane selectivity to cations can be rationalised in terms of binding to fixed negative sites within the membrane. The driving force underlying selectivity is suggested to be determined by the free energy difference between cation/site and cation/water interactions.

Generally, the cation preferred by a membrane bound negative site will be the one that produces the greatest decrease in free energy on changing its interaction with water to that negative site. This may be expressed by the equation,

$$\Delta Fa, site - \Delta Fb, site - \Delta Fa, water + \Delta Fb, water \quad \ldots\ldots\ldots (1)$$

The relative affinities of the site for two different cations a and b will be governed by the free energy difference where ΔFa, site and ΔFb, site are the free energies of interaction between the cation and the negative membrane bound site; and ΔFa, water and ΔFb, water are the free energies of hydration.

Two extreme cases may arise, the first is that the electric field strength of the negative bound site may be high, and so the term ΔFa, site - ΔFb site may dominate equation (1). At present there are no readily-available data concerning the free energies of site interaction for various metals, mainly because it is not certain what chemical groups are involved in the formation of membrane bound negative sites. The other extreme may arise when the free energies of hydration are high and in that instance the term ΔFa, water + ΔFb, water will dominate equation (1), and will be the determining factor in site binding. Table 6.2 displays the enthalpy of hydration for some of the metals investigated. It can be seen that there is no apparent correlation between order of effect on lead transport and enthalpy of hydration.

Table 6.2. The enthalpy of hydration of selected metal cations

Order of potency on lead transport ←

Metal Cation	La	Zn	Ce	Fe	Sr	Cd	Be	Al	Cr	Ni
Δ Hhyd ($k J mol^{-1}$)	–	2056	–	1908	1485	1805	–	4690	1835	2090
	GROUP THREE						GROUP TWO			GROUP ONE

The thermodynamic approach postulated by Diamond and Wright (1969) has been demonstrated to hold for alkali and alkaline earth metals, but cannot predict or explain competition effects for metals of higher mass or complexity. A possible reason for this departure from the proposed theory could be that induction effects caused by the presence of metals at high concentrations, alter the field strength of the negative site, and thus the relative balance between ion/site and ion/water energies will be shifted either way and will make predictions difficult.

6.5. CONCLUSIONS

It is clear that neither ionic radii, hydrated ion size or thermodynamic considerations can satisfactorily explain the experimental data. The postulation proposed by Frizzel and Schultz (1972) that the tight junctions of leaky epithelia act like ion-exchange columns, however, may be a valid concept. Ion selectivity sequences for general purpose ion exchangers (strong acid - SO_3H or weak acid - CO_2H cation exchangers) have been published, (Gordon and Ford, 1972), and Table 6.3. displays some of the major trends. It can be observed that for the first divalent metal sequence, lead will bind most tenaciously to an ion-exchange column, and therefore other metals will displace lead if present at higher concentrations. The experimental data demonstrates that the rate of lead cation transfer is not significantly affected until a second metal cation is present at great excess, (Figures 6.1 to 6.5

Table 6.3 ION AFFINITY SEQUENCES

For dilute aqueous solutions at room temperature (in order of most to least strongly held)

Metal species

M^{+}	Ag > Tl > Cs > Rb > NH_4 > K > Na > Li > R_4N
M^{2+}	Pb > Ba > Sr > Ca > Mg > Be
M^{2+}	Zn > Cu > Ni > Co > Fe > Ba > Sr > Ca > Mg
M^{3+}	La > Ce > Pr > Nd > Pm > Sm > Eu > Gd > Tb > By
	Y > Ho > Er > Tm > Yb > Lu > Sc > Al
Mixed	Fe^{3+} > Al^{3+} > Ca^{2+} > Mg^{2+} > K^{+} > Na^{+} > H^{+} > Li^{+}

respectively).

Previous data (Chapter Five) suggests that lead transfer proceeds via a paracellular route which incorporates the tight junctions as the rate-limiting step. It is concluded that lead transport is initiated by lead cations binding to anionic fixed sites, and their progressive movement from one site to another down the tight junction. The driving force for the process is the lead concentration gradient across the intestinal epithelium. The presence of other metal cations may cause a reduction in lead cation transfer when present at high concentrations by either 1) competing for the anionic sites within the tight junctions, or 2) contending for entry into the tight junctions from the bathing medium, or possibly a combination of both.

6.6. THE EFFECT OF DIETARY METALS ON THE UPTAKE OF LEAD BY INTESTINAL TISSUE

The effect of the ten metals (Ni, Be, Cd, Sr, Zn, Cr, Al, Fe, Ce and La) on the uptake of lead by intestinal tissue were investigated concurrently with their effect on lead transport. In addition, the effect of mercury was investigated. Table 6.4 demonstrates various effects produced by the presence of other metals on lead uptake by intestinal tissue; some produce increased uptake, some produce no effect, and some produce decreased lead uptake. Those metals that either elevated or decreased lead uptake by the tissue were further investigated over a broad concentration range (10^{-6}-10^{-1} M).

Table 6.4.

THE EFFECT OF VARIOUS METALS ON THE UPTAKE OF LEAD BY INTESTINAL TISSUE

Metal ion (present at 10^{-2} M)	Tissue lead uptake (nMg^{-1})	Significance of deviation from control value
Ni	3.8 ± 0.2	N/S
Be	4.0 ± 1.0	N/S
Cd	5.4 ± 1.1	$p < 0.05$
Sr	3.8 ± 0.9	N/S
Zn	7.0 ± 0.7	$p < 0.001$
Hg	4.5 ± 0.8	N/S
Cr	3.6 ± 0.7	N/S
Al	6.2 ± 0.8	$p < 0.001$
Fe	1.6 ± 0.3	$p < 0.01$
Ce	3.2 ± 0.9	N/S
La	3.8 ± 1.0	N/S
Control	3.6 ± 0.5	N/S

Each value is the mean of six experimental observations $\pm$ standard error of the mean

The lead uptake by intestinal tissue in the presence of either zinc, cadmium, aluminium, mercury or iron was investigated as previously described, (Section 6.3). There was a significant elevation of lead uptake in the presence of all the metals, but at different concentrations; and in the presence of two of the metals, lead uptake was significantly lowered. The details are as follows;

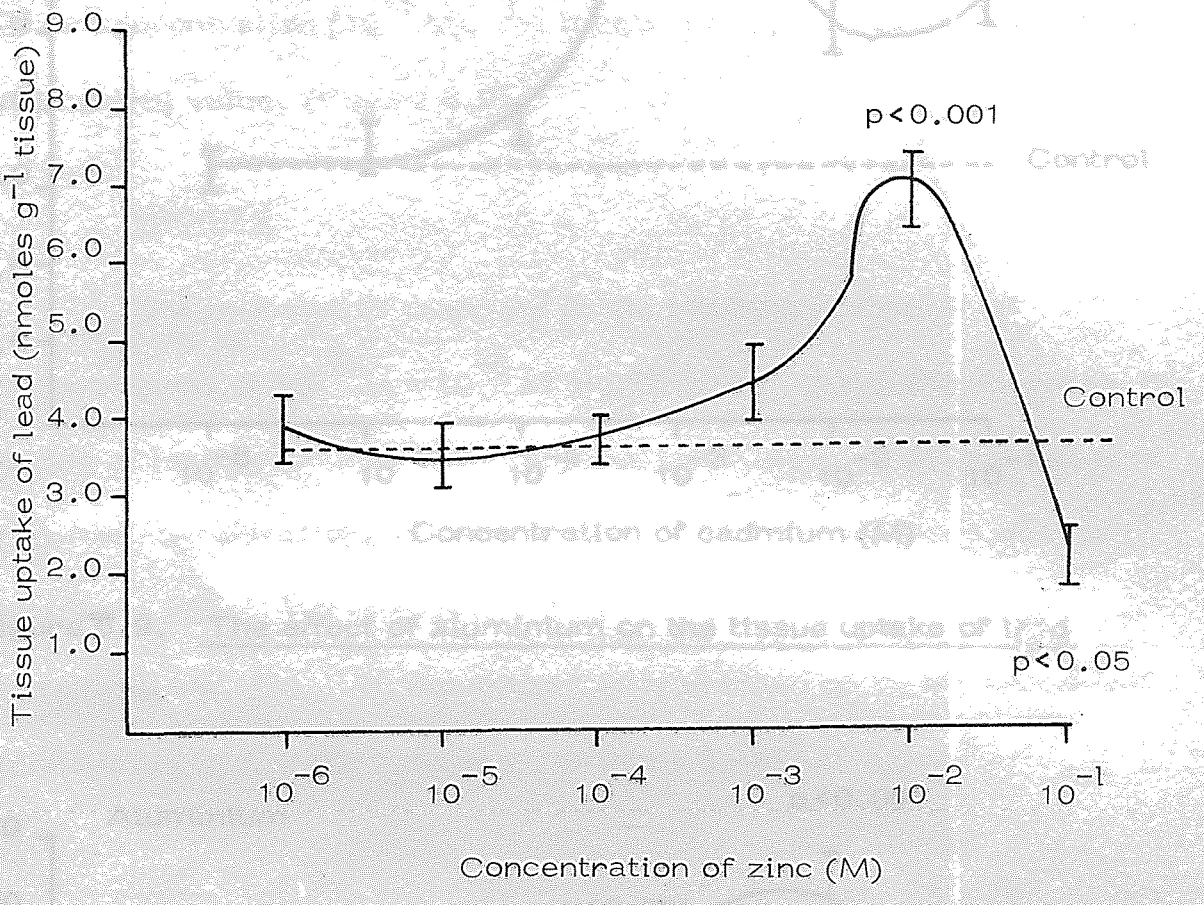
Zinc

The amount of lead taken up by intestinal tissue was little affected by the presence of zinc in the incubation medium at concentrations between 10^{-6} - 10^{-3} M. However, when zinc was present at a concentration of 10^{-2} M, there was a significant elevation ($p < 0.001$) of lead taken up by the tissue, (Figure 6.7). Further, when zinc was present at a concentration of 10^{-1} M, there was a significant decrease ($p < 0.05$) in the amount of lead taken up by the intestinal tissue compared to a control value.

Cadmium

The presence of cadmium (10^{-6} M - 10^{-4} M) in the incubation medium caused little effect upon the tissue uptake of lead. However, in the presence of higher concentrations of cadmium (10^{-3}M - 10^{-1} M), there was a significant elevation ($p < 0.05$) in the amount of lead associated with the tissue, (Figure 6.8).

Figure 6.7. The effect of zinc on the tissue uptake of lead



Each value is the mean of six experimental observations ± standard error of the mean

Figure 6.8. The effect of cadmium on the tissue uptake of lead

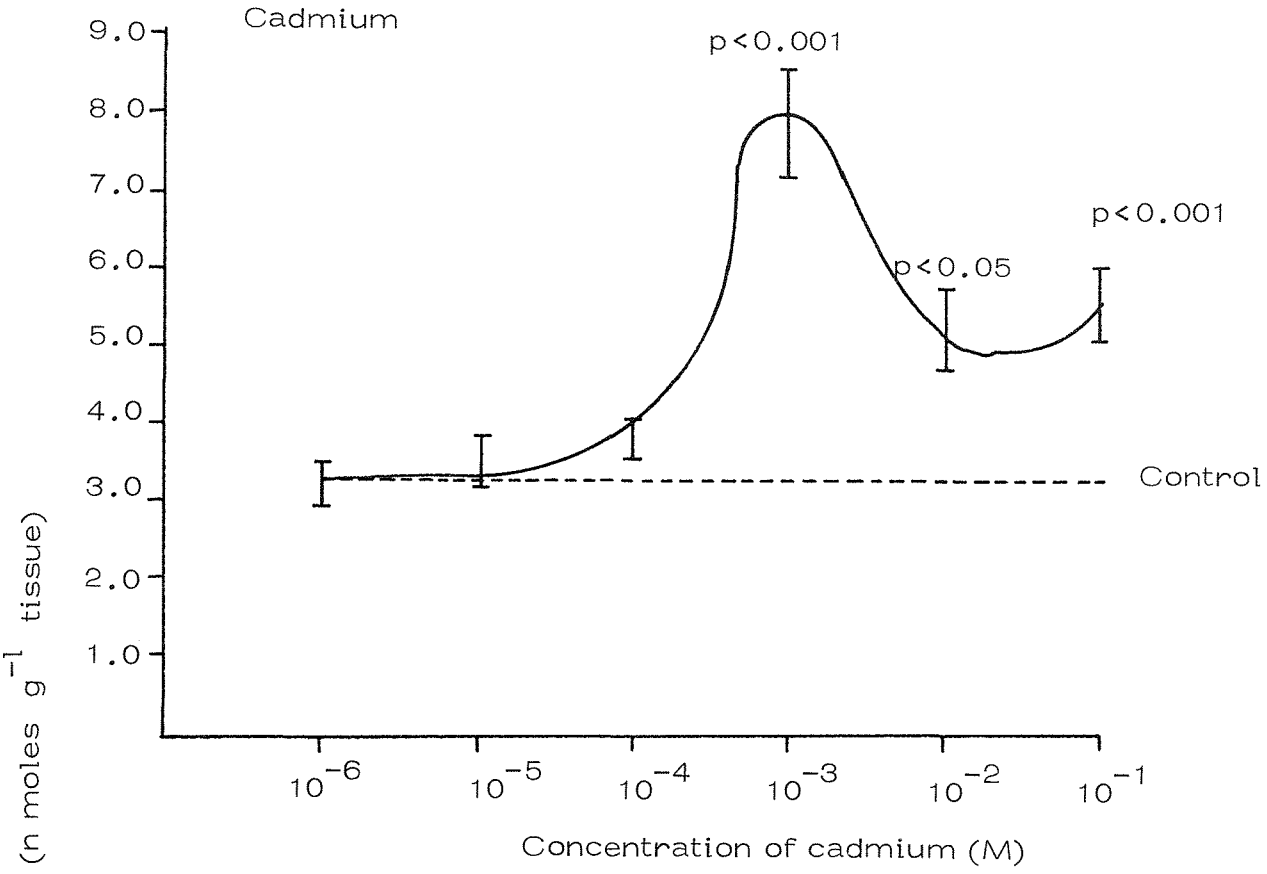
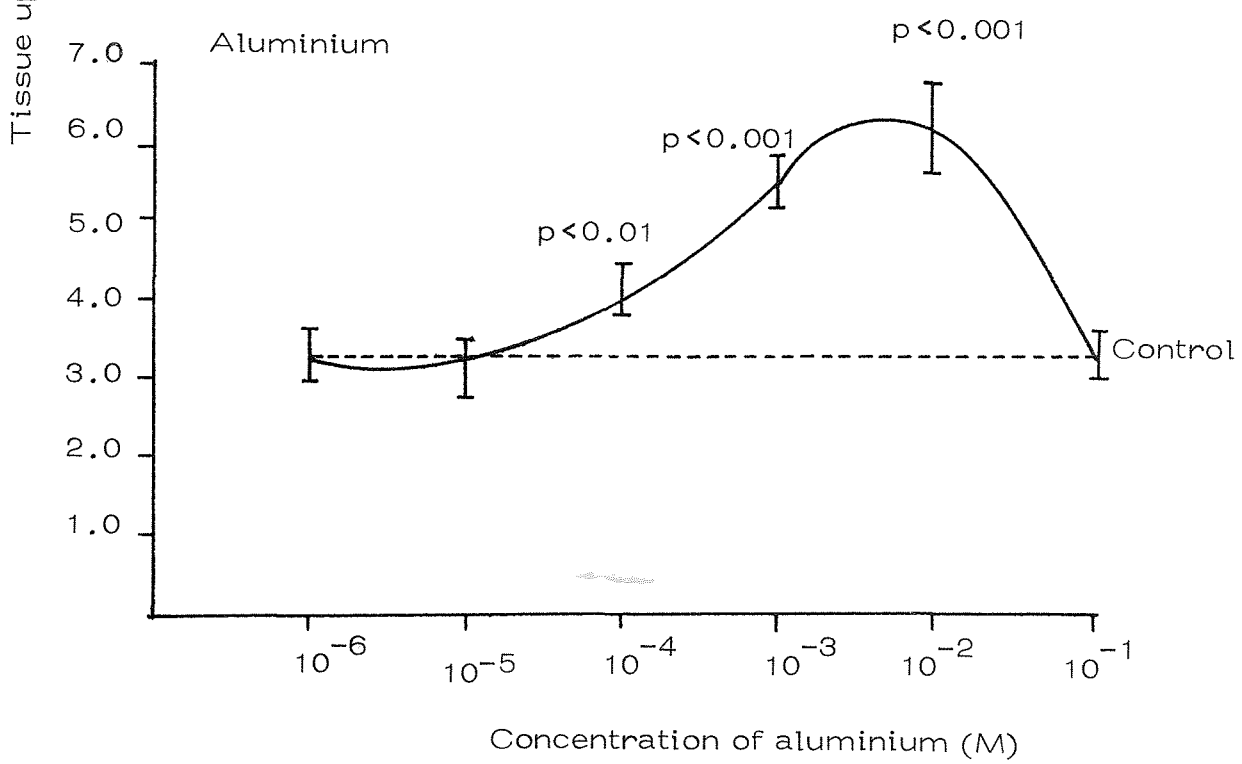


Figure 6.9. The effect of aluminium on the tissue uptake of lead



Each value is the mean of six experimental observations $\pm$ standard

Aluminium

The presence of aluminium (10^{-4} M - 10^{-2} M) in the incubation medium caused a significant ($p < 0.01$) elevation of lead taken up by intestinal tissue. However, when aluminium was present at a higher concentration (10^{-1} M), the tissue lead uptake value was the same as a control value, (Figure 6.9).

Mercury

The presence of mercury in the incubation medium at concentrations of 10^{-6} M - 10^{-5} M significantly ($p < 0.05$) decrease the amount of lead associated with the intestinal tissue. In the presence of higher concentrations of mercury (10^{-4}M - 10^{-2} M) there was no significant effect, but when present at even higher concentrations, (10^{-1} M) the amount of lead taken up by the tissue was significantly ($p < 0.001$) elevated, (Figure 6.10).

Iron

The presence of iron in the incubation medium at concentrations of 10^{-6}M- 10^{-4}M caused a progressive increase in tissue lead uptake, which became significantly elevated ($p < 0.02$) above a control value at an iron concentration of 10^{-4} M. Subsequent increases of the iron present (10^{-3} - 10^{-1} M) produced a progressive decrease of tissue associated lead, which became significantly lower ($p < 0.01$) than a control value in excess of 10^{-2} M iron, (Figure 6.11).

Figure 6.10 The effect of mercury on the tissue uptake of lead

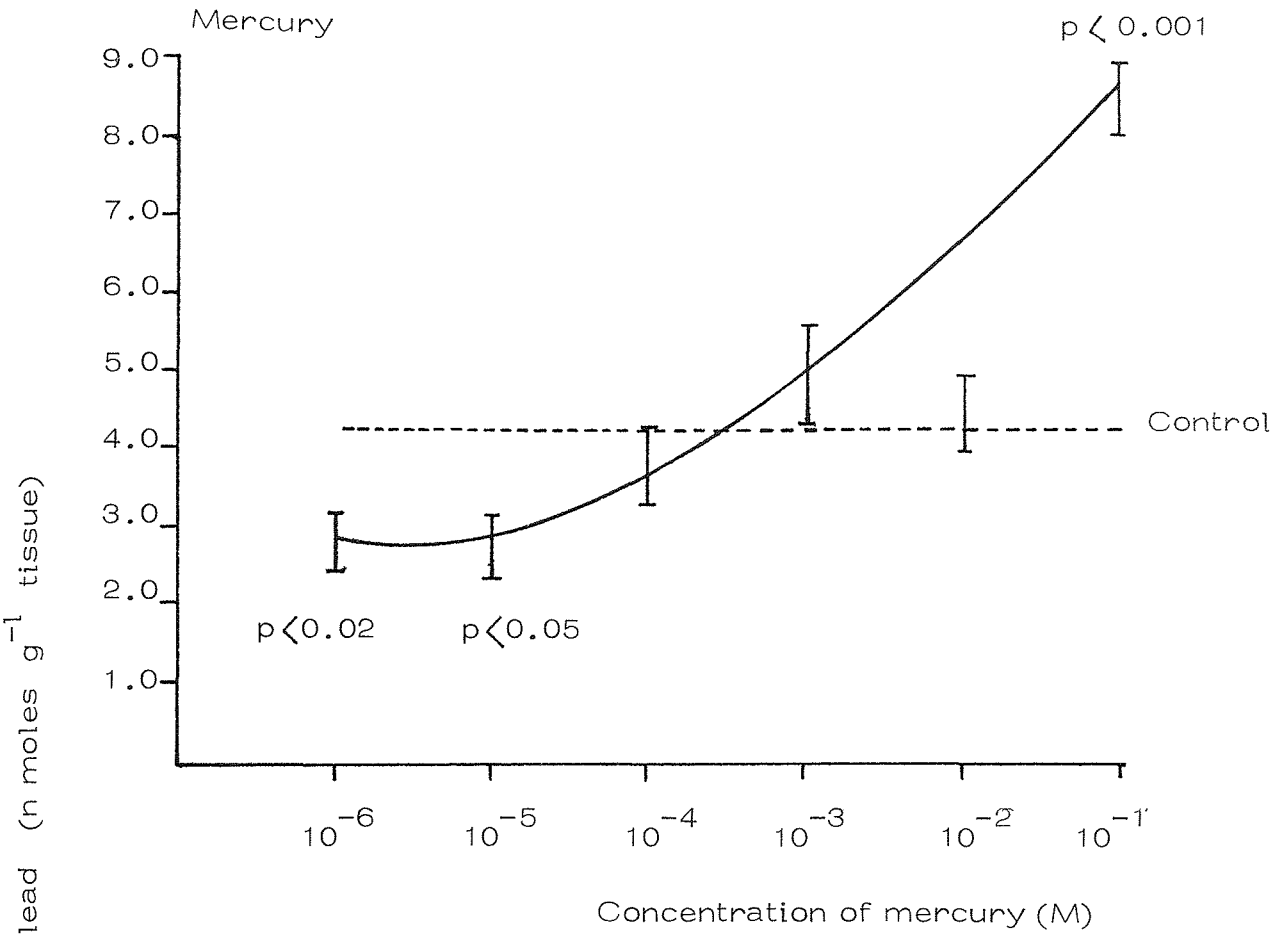
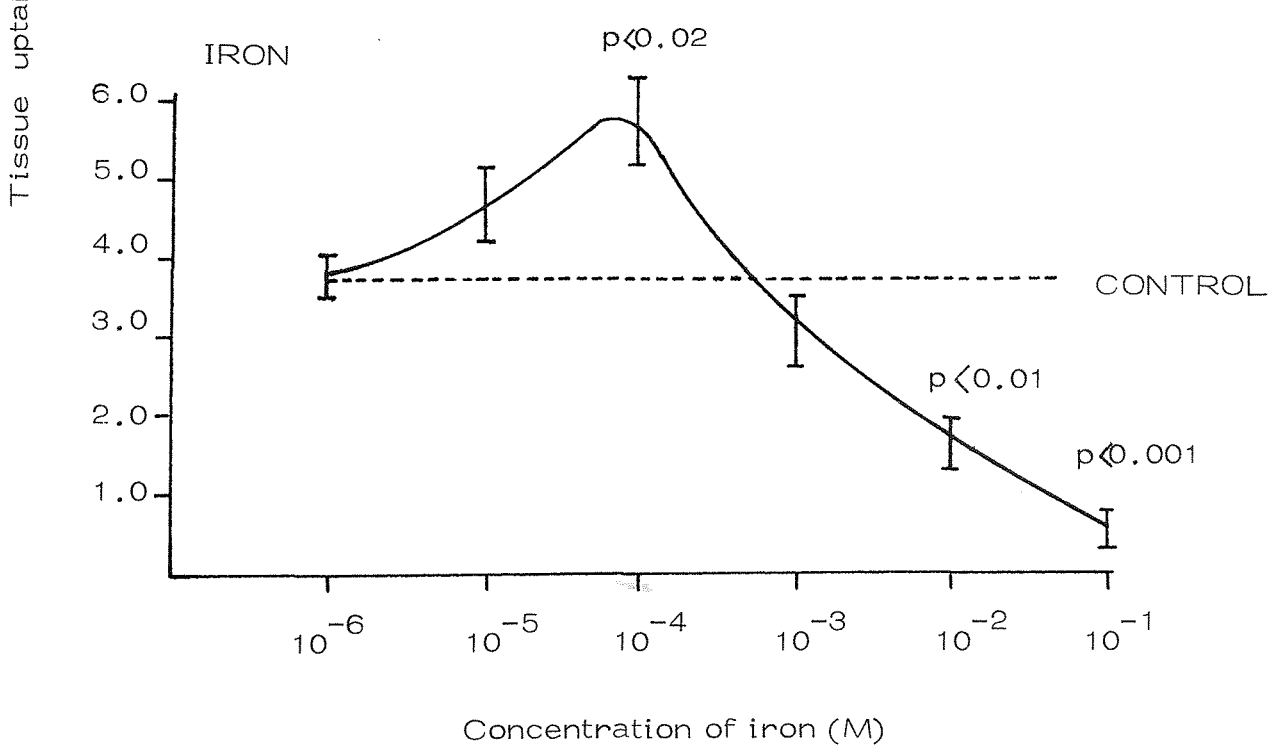


Figure 6.11 The effect of iron on the tissue uptake of lead



Each value is the mean of six experimental observations $\pm$ standard

6.7. DISCUSSION

A limited amount of data has been published on the effect of the presence of one metal on the tissue uptake of a second metal. Sahagian et al., (1971) conducted experiments on the uptake of zinc, cadmium and mercury, but not lead, using intestinal strips. However, the data are of limited value since the technique used exposed both serosal and mucosal surfaces of the intestinal tissue to the metal under investigation, hence the data may be of a combined uptake. Sahagian et al., (1971) report that the tissue uptake of any one metal investigated can greatly influence the uptake of a second, particularly when the concentration of one metal approaches and exceeds the concentration of the second. Unfortunately, the authors do not postulate a mechanism that explains their reported observations.

The experimental data presented in Figures 6.7 to 6.11 may be interpreted in terms of two possible mechanisms. The first mechanism is a process of metal binding to the tissue surface which at high concentrations of metal cations induces membrane permeability changes, which subsequently cause metal cations to enter cells and bind at intracellular sites. Thus lead cations could enter the interior of epithelial cells, along with the second metal cations, and bind to the cell contents and organelles. At relatively higher metal cation concentrations lead cations may be displaced from binding sites both on the exterior cell surface and within the cell, which may explain the

characteristic decreases in lead uptake exhibited by zinc and iron. The concept of metal cation induced increases in cell membrane permeability, however, is difficult to rationalise in the light of the present knowledge of membrane function. An alternative explanation of the experimental data is that of coprecipitation of lead with a metal phosphate at the tissue surface.

Heavy metal salts are generally hydrolysed to insoluble compounds in slightly acidic solutions. The glycocalyx, and its associated acid microclimate, (see Section 4.8, Chapter Four) at the tissue surface could provide the acidity necessary for the precipitation of insoluble basic salts and colloids which may have a charged surface. The glycocalyx region is also rich in phosphate ions as a consequence of A.T.P. hydrolysis. A substantial amount of metal phosphate could therefore be produced, and if so, it is likely that it would be precipitated at the tissue surface. The prevailing conditions would then be those of ion-exchange at the charged surface of the precipitate.

The basic principle of coprecipitation is; when a substance in solution becomes insoluble, through chemical reaction and precipitates out of solution, (thus forming the macrocomponent), it may carry other substances that are present in trace amounts, (the microcomponent) alone with it. There are two types of coprecipitation that may occur. The first form involves the coprecipitation of a

trace substance when it has a similar size and charge that enable it to enter the normal crystal lattice formation of the macrocomponent; that is, it forms crystals that are isomorphous with those of the macrocomponent. The second type of coprecipitation involves ion-exchange, or other chemical or physical adsorption of the microcomponent on to the surface of the precipitated macrocomponent.

The variation of charge and ionic radii of the metals investigated in this chapter cannot be correlated with the elevation in tissue uptake of lead (see Table 6.1). It is therefore unlikely that isomorphous replacement coprecipitation is the mechanism involved in this instance. Adsorption coprecipitation, however, is a probable candidate, and the experimental data can be rationalised by such a process.

In conclusion, the data presented in Figures 6.7 to 6.11 demonstrate enhanced tissue lead uptake and may be explained by the adsorption of lead cations onto the surface of metal phosphate precipitates, which may be present at the tissue surface at relatively high concentrations of zinc, cadmium, aluminium, mercury and iron. The relative reduction of tissue lead uptake after a maxima has been reached, is indicative of competition between lead cations and the second metal cations, for binding sites on both the tissue surface and on the surface of the precipitated metal phosphates. The implications of increased tissue uptake of lead cations are further discussed in Chapter Seven.

CHAPTER SEVEN

AN ASSESSMENT OF THE EXPERIMENTAL DATA

Data obtained from *in vitro* experimentation described in this thesis, can be used to assess three possible models proposed in the scientific literature which perport to describe lead transport. The possible models are

1) an active carrier mediated transcellular transport of the lead cation,

2) a passive diffusion of lead cations via a paracellular route, and

3) the transfer of some lead by a non-specific lead carrier, together with an element of passive diffusion of lead cations.

7.1. GENERAL PHYSIOLOGICAL CONSIDERATIONS

The use of an *in vitro* technique in these studies was advantageous as the chemical form of the transported species, and physiological and biochemical parameters could be strictly controlled. The use of balance studies, which many investigators favour, do not allow the effects of individual chemical or physiological parameters to be investigated, but instead produce kinetic data which describe overall transport characteristics which may be the summation of various antagonistic effects. It must be remembered, however, that *in vitro* techniques may underestimate the rates of absorption of a substance, since materials that would under normal conditions be removed in the blood stream, would with an *in vitro* technique such as the everted sac

not be removed. Instead, after a few minutes incubation a concentration of a solute is built up in the serosal compartment and muscle layers, (see Pritchard and Porteous, 1977). A possible effect of solute build up could be to produce a false saturation phenomena. As the experimental data outlined in Chapter Four demonstrated that the transfer of lead was extremely slow, it means that there was no substantial concentration build up of lead in the serosal compartment and underlying muscle layers.

The in vitro everted sac technique used throughout these experimental investigations were demonstrated to be capable of maintaining various physiological functions, namely;

a) producing a linear rate of water transport, by producing a steady transmural potential difference,

b) actively accumulating glucose and an amino acid.

The structural integrity of the intestinal preparations was also demonstrated to be maintained, and thus it was concluded that the technique produced viable tissue preparations, suitable for transport studies.

Five aims were established in Section 1.6 of Chapter One and the extent to which each has been fulfilled will now be considered and discussed.

7.2. THE DEPENDENCE OF LEAD TRANSPORT UPON METABOLIC ENERGY

The kinetic data presented in Chapter Four (Section 4.2) clearly demonstrates that the transfer of the lead cation is independent of mucosal temperature and glucose concentration. It is also independent of an oxygen requirement and the general metabolic processes of the small intestine. The observations have been interpreted to suggest that lead cation transport across the intestinal epithelium occurs by a mechanism of passive diffusion. Gruden and Stantic (1975) and Coleman (1979) have published data which lend support to this conclusion.

The data however, stand in contradiction to those of Aungst and Fung (1981) and Barton et al., (1978). The former group have suggested an active transport mechanism, and the latter have suggested a carrier mediated form of transfer for the lead cation. It is demonstrably clear that the experimental conditions used by Aungst and Fung (1981) do not ensure that the lead cations remain soluble. The practice of gassing lead solutions with carbon dioxide will induce the formation of lead carbonate which is not soluble above a concentration of approximately 5×10^{-6} M, (Linke, 1965). The implications of the conclusions drawn by Barton et al., (1978) will be fully assessed in Section 7.3.

Coleman (1979) in his conclusions regarding the energy

dependence of lead transport suggested that the cations transfer was linked to a concomitant movement of water. Although a good correlation between lead cation and water transport can be demonstrated, it is most likely that it is not a causal relationship, as lead is transported independently of water movement when fluid movement is reversed.

7.3. THE ROUTE FOR LEAD TRANSFER

The lead cation must translocate the intestinal epithelium by one of two routes, either between the cells (the paracellular route), or across the lipid membrane into the cell (the transcellular route).

In Chapter Five the presence of a number of methylxanthines were demonstrated to be capable of significantly reducing the rate of lead cation transport. The effect of methyl xanthines is known to induce cell swelling and consequently to obliterate the lateral intercellular spaces that constitute part of the paracellular route. Hence, it is concluded that lead cation transport proceeds to traverse the intestinal epithelium by movement through the lateral intercellular spaces, and therefore via the paracellular route.

A transcellular route of lead cation transport has been proposed by Barton et al., (1978), who propose that lead cations bind to a protein carrier that subsequently translocates them across the

absorptive cell. The major evidence in support of a protein carrier is derived from chromatographic data which indicates that lead may be predominantly associated with a vitamin D-dependent calcium binding-protein (CaBP). However, it cannot be stated with certainty that the lead/calcium binding-protein interaction is not an artefact of the protein extraction process. Mahaffey et al., (1979) have demonstrated that it is the luminal presence of a low calcium concentration, and not a state of calcium deficiency per se, that causes an increase in lead transport; thus refuting the specific role played by calcium binding-proteins in lead transport. Moreover, Mykkänen and Wasserman (1981) conclude that there is no evidence of competition between lead and calcium ions at the rate-limiting step of absorption in the small intestine. It is possible that all cations may utilise the paracellular route of transport, and indeed the notion of a "common route" for the transfer of lead and other minerals has been suggested (Meredith et al., 1977), although the location of such a route was not stated or expounded in any detail.

The experimental data presented in Chapters Four and Five, and a reinterpretation of the data reported by other authors leads to the conclusion that lead ions are transferred across the intestinal epithelium by a mechanism of passive diffusion which utilises a paracellular route. Thus two aims of the present research that were established at the initiation of the experimentation have been accomplished.

7.4. THE INTERACTION OF LEAD CATIONS WITH THE INTESTINAL TISSUE SURFACE

The data presented in Chapter Four (Section 4.5 and plates 5 to 13 respectively) demonstrate that a surface adsorption phenomen occurs whereby lead cations are rapidly adsorbed onto the tissue surface. Coleman (1979) observed a similar occurrence, but his methodology only permitted lead uptake to be investigated at concentrations upto 5×10^{-5} M. In the present investigation the intestinal tissue demonstrated a vast capacity to adsorb lead cations, which only became limited at luminal lead concentrations in excess of 10^{-2}M. It is the vast capacity to adsorb lead cations that precludes the possibility of the binding sites being proteins of the type described by Barton et al., (1978). Blair et al., (1979) has suggested the identity of the binding sites to be phosphate anions generated by the activity of brush border A.T.P-ases, (see Section 4.8, Chapter Four). Lead cations on interaction with phosphate anions would be sequestered in a covalent form as lead phosphate onto the tissue surface. Estimations of the phosphate anion concentration at the tissue surface remain obscure, but it is known that A.T.P-ase activity is high in the rat intestinal brush border, (Fujita et al., 1971). The creation of a tissue surface acidity of pH 3.5 (Blair and Matty, 1974) would in consequence produce a concurrent phosphate anion concentration of approximately 5×10^{-3}M.

An alternative source of phosphate groups could be the phospholipid consititnent of epithelial cell membranes. An estimation of the membrane phosphate can be made on the follow assumptions; phospholipids constitute approximately 26% by weight of typical cell membranes (Dewey and Barr, 1970); average phospholipid molecular weight is 900 (Lehninger, 1976), and tissue density is the same as water. Hence;

$$\frac{\frac{26}{100} \times 1000}{900} = 0.289 \text{ M.}$$

The concentration of lead at the tissue surface may be calculated in a similar manner; maximum lead bound = 41 μmoles (table 4.4); membrane surface accounts for 10% of tissue mass, therefore, 41 X 10^{-6} moles g^{-1} with 0.1 g of membrane = 41 X 10^{-2} moles /1000 g = 0.41 M. Thus there appears to be sufficient phosphate present to account for the lead binding observed.

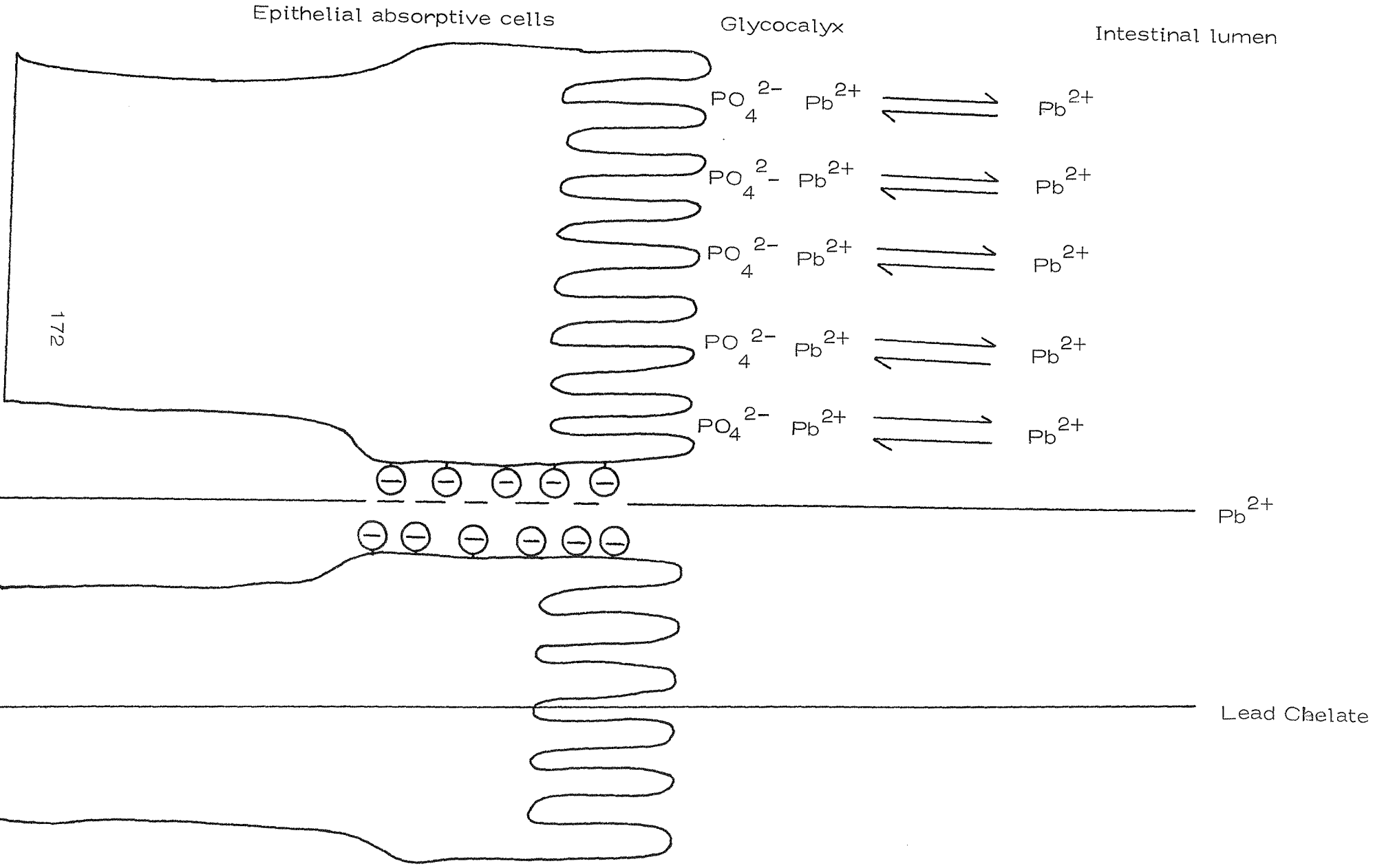
7.5. THE CONSTRUCTION OF A CONCEPTUAL MODEL TO DESCRIBE LEAD TRANSPORT BY THE SMALL INTESTINE

The experimental data obtained in this thsis can be used to construct a conceptual model that describes lead transport by the small intestine. The model consists essentially of two processes acting in concert; the rapid binding of lead cations to the tissue surface, and the concurrent slow passive diffusion of lead cations from the gut lumen across the epithelium to the serosal side of the intestinal preparation.

The diffusion of lead cations proceeds via a paracellular route that incorporates the tight junction, which act as an ion-exchange column, and the lateral intercellular channels. The size of the tight junctions and the lateral intercellular channels, and the charge associated with the tight junctions are the rate-limiting factors in determining lead transport. Hence the prevailing conditions of luminal ionic composition, pH or the presence of methyl-xanthines are critical.

The second process involves a large rapid interaction of lead cations with negative groups on the tissue surface which temporarily immobilises the lead, (see Section 4.1). The chemical identity of the tissue surface binding groups are most likely to be phosphate anions either loosely associated with, or integral components of the phospholipid apical epithelial cell membrane. The extent of lead cation interaction with the intestinal tissue surface will be determined by a "physico-chemical" equilibrium that allows approximately 10-20% of the total lead ions present to interact. Thus any factor that increases the number of lead binding groups (sites), such as anoxia or metabolic inhibitors (see Section 4.2 and 4.3) will move the equilibrium towards lead binding and will therefore, result in less lead cations being available in the gut lumen to diffuse across the intestinal epithelium, (Figure 7.1). Further, as unbound lead cations move down the tight junctions and diminish the luminal

The model proposed to describe the transport of lead cations by the small intestine



concentration of lead cations, there will be a slow release of lead cations from the tissue into the intestinal lumen, thereby providing a constant supply of lead cations for transport by diffusion.

7.6. RATIONALISATION OF THE DATA IN THE LITERATURE IN TERMS OF THE MODEL

Any model which adequately describes the transport of lead into the body, should also be able to explain why so many of the nutritional and physiological factors (see Table 1.2, Chapter One) apparently influence lead absorption. An attempt to explain some of the literature cited data in terms of the proposed model is now possible.

The effect of metals

The reported antagonism between calcium or iron absorption and the absorption of lead, (Ledered and Bing, 1940; Mahaffey et al., 1970; Gerber and Deroo, 1975; Quarterman and Morrison, 1975; Barltrop and Khoo, 1976, and Six et al., 1972; Ragan, 1977; Hamilton 1978; Barton and Conrad, 1978, Flanagan et al., 1979 and Barton et al., 1981, respectively), may be explained in the following way. Lead cations utilise a passive paracellular route of transport, whereas calcium and iron ions predominantly utilise an active, carrier mediated transcellular route of transport. In circumstances of excess dietary calcium or iron levels, the active transport system becomes saturated. The calcium and iron

that remain in the intestinal lumen, move down the tight junctions and thus in effect compete with the lead cations for the paracellular route, and thus reduce the rate of lead transport. Conversely, low levels of dietary calcium and iron ensures that all the available calcium and iron cations are actively transported by their respective carriers, and thus few are available for transfer by the paracellular route, and consequently lead transport is enhanced. Dietary metals that do not have a specific carrier mediated transport system, such as zinc (Cerklewski and Forbes, 1976) phosphorous (Quaterman and Morrison, 1975) and magnesium (Fine et al., 1976) probably exert their depressive effects on lead transport by direct competition for the paracellular route of transport.

The effect of chelating agents

Most of the experimental investigations conducted in this thesis were concerned with the lead cation. However, under normal physiological conditions it is possible that metals are present in the intestinal lumen as complexes or chelates as well as cations. The amount of a metal complexed depends on its stability constant and on interluminal factors such as pH and on the concentration of other competing metals and ligands. Generally, as a result of chelate formation the properties of metals are masked by the ligands they complex with, and the rate of transfer of the complex depends upon its physicochemical properties, e.g. the lipid solubility and

electric charge. The molecular size and chemical properties of most metal chelates suggests that passage of chelates can occur through both the "non-porous" lipid portion of the cell membrane, as well as through the protein portion. Thus one may expect to observe different transport kinetic characteristics with lead chelates than with lead cations.

The experimental data presented in Section 4.7 demonstrate that a much greater rate of lead transport is possible with chelated lead species, and that the characteristic lead uptake by the intestinal tissue, observed with lead cations, is not reproduced by lead chelates. The chelated lead species investigated are not positively charged (see Table 7.1) and would not therefore interact with the intestinal tissue surface in the same manner as lead cations. The low tissue uptake value obtained when lead is chelated may indicate the presence of the lead chelate in the mucosal extracellular space. It is possible that due to their size and charge lead chelates passively diffuse across the epithelial cell membranes and thus adopt a trans-cellular route of transport. The data displayed in Table 7.1 suggests that species which have an overall double negative charge move more rapidly than those which have either a single or triple negative charge, while those species that are neutral traverse the epithelium least rapidly.

The presence and absence of food in the gut lumen

Several authors have reported a significant increase in lead

Table 7.1. THE EFFECT OF CHARGE AND MOLECULAR WEIGHT ON THE RATE OF CHELATED LEAD TRANSPORT

Reacting group	Molecular weight of lead species	Charge associated with the lead species
E.D.T.A.	[Pb-E.D.T.A]= 495	2-
N.T.A.	[Pb-N.T.A.)= 395	1-
D.T.P.A.	[Pb-D.T.P.A]=595	3-
Ascorbate	[Pb-Ascorbate$_2$]=561	0

Decreasing rate of transport

E.D.T.A. = Ethylenediaminetetraacetic acid

N.T.A. = Nitrilotriacetic acid

D.T.P.A. = diethlenetriaminepenta acetic acid

Table 7.1. is taken from the experimental data presented in Figure 4.10 of Section 4.7. (Chapter Four).

absorption in both human subjects and rats which were dosed with lead after a period of fasting, (Garber and Wei, 1974; Quarterman et al., 1976; Conrad and Barton, 1978 and Chamberlain et al., 1978). This observation may be explained by the factors that essentially increase the availability of lead. The three factors that reduce lead availability in the presence of food are a) decreased solubility of lead by formation of inorganic lead precipitates, b) co-precipitation with insoluble food substances, and 3) decreased gut retention times.

a) It is reported (Heard and Chamberlain, 1981, personal communication) that in human subjects the absorption of a dose of lead was reduced by a factor of 1.2 by the addition of calcium, and by a factor of 1.4. by phosphate, but by a factor of 6 when calcium and phosphate were added together. The authors conclude that calcium carbonate and sodium hydrogen phosphate reduce lead uptake by rendering it less soluble, probably by co-precipitation of the phosphate.

b) Wise and Gilburt (1981) conducted investigations with rats and report that lead is precipitated in the gut lumen by phytate, (which is present in the normal diet), in the presence of calcium. Below pH 4 calcium phytate is soluble and therefore, lead in the stomach would be soluble. However, at pH 5 or above, the calcium phytate complex is insoluble and provides a large surface for the binding of lead, thus rendering lead cations unavailable for inter-

action with intestinal tissue or passive diffusion. It should be noted that the co-precipitation of lead with a calcium-phytate complex referred to above, is distinctly different to the co-precipitation of lead with metal phosphates on the tissue surface (described in Section 6.8). Lead cations coprecipitated in the intestinal lumen would be unavailable for transport unless the lead-calcium-phytate complex was resolubilised due to a change in pH. In the latter case, the lead cations are precipitated on the tissue surface and would be potentially available for exchange with the intestinal lumen, and thus may be important in lead absorption.

c) The presence of food in the intestinal tract provides a stimulus for the process of peristalsis, (McDowall, 1960). peristalsis consists essentially of a progressive wave of contraction which is preceded by a wave of relaxation, the resultant effect moves partially digested dietary material along the intestinal tract. Absence of food reduces peristalsis, and thus increases the gut retention time of a given dose of lead. Consequently, more of the dose will be absorbed by the intestinal tissue, and therefore available for exchange with the lumen and potentially for diffusion across the intestinal epithelium.

It is clear that the presence of food in the gastrointestinal tract, will cause all three of the above factors to act in unison to reduce the availability of dietary lead to the absorptive mechanism

described in Section 7.5.

Miscellaneous factors

It has been extensively observed that the young of various animal species have a greater capacity to absorb lead. It has been postulated (Keller and Doherty, 1980) that a reason for increased lead absorption may be the prevailence of pinocytotic mechanisms in young animals, which become absolete when the animal matures. The accumulation of lead cations onto the intestinal tissue surface may be a factor in initiating pinocytotic activity and hence, increased lead transfer. However, there is little evidence to suggest that the process of pinocytosis is a major contribution to lead absorption. It is also reported that the tight junctions in neonates are larger than those found in adult mice, and are therefore capable of greater rates of transport via the paracellular route. This fact may be responsible for the increased rate of lead transfer that is presently attributed to the action of pinocytosis in the young.

Coleman (1979) has reported that the absence of calcium ions is a factor in increasing tight junction size, and may therefore affect lead transport. The effect of age and the absence of calcium ions may fit into the postulated model of lead cation transport, since both are factors that alter the permeability of tight junctions, and consequently increase the rate of lead transport via the paracellular route.

7.7. CONCLUSIONS

The objectives for this work set out in Section 1.6 have been accomplished. Definitive experimentation has yielded information used to construct a model of lead cations transport, which can be used to rationalise some of the data of other workers. It is however, inadvisable to extrapolate a model based essentially upon data obtained from the rat, to the human situation. Whilst the anatomy and physiology of the rat small intestine have similarities to the human small intestine, care must be taken when making comparisons. Consequently, the model may serve as a starting point to organise a strategy for subsequent investigations upon human subjects.

Such studies may include the following lines of investigation.

1) Research into the absorption of adventitious sources of lead during the fasting state. It is known that dust particles contain several hundred p.p.m. of lead, and that if ingested after a period of fasting, (e.g. overnight), it is possible that upto 40% may be absorbed into the body.

2) Chamberlain et al., (1978) report that a large variation of lead absorption occurs (24-60%) during the fasting state in apparently normal subjects. The data may indicate the possible existence of "natural susceptibility" which may have a physiological or even genetic basis. Such variations should be further investigated with a view to devising a system of screening for potential workers in the lead industry.

3) The role of the acid microclimate in lead absorption may be substantiated or refuted, by conducting investigations on human subjects who have intestines in a diseased state. Such states may include coeliac or Crohn's disease where the glycocalyx is diminished; blind loop syndrome where the glycocalyx is affected due to excessive deoxycholate production; and conditions where the brush border enzymes (particularly phosphatases) are impared.

4) Determination of diets which would be advantageous in reducing lead absorption, which could be recommended for susceptible groups within the general population. Such groups may be young children, workers in the lead industries and subjects who are iron-deficient anaemic.

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