STUDIES OF THE EXCRETION OF ALUMINIUM BY THE KIDNEY

AND

THE TOXIC EFFECTS OF THE ELEMENT ON DNA

Felix Salvador Emilio Monteagudo

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ABSTRACT

THESIS TITLE: Studies of the excretion of aluminium by the kidney and the toxic effects of the element on DNA.

CANDIDATE: Felix Salvador Emilio Monteagudo, c/o Department of Pharmacology, University of Cape Town, Cape Town. February, 1991.

Aluminium is an element of increasing clinical importance. It not only has uses as a medicinal substance but also in recent years it has been shown to be the cause of considerable toxicity, particularly in the setting of chronic renal failure. Diseases that have been shown to be associated with aluminium, or in which it has been implicated, include dialysis dementia, renal osteodystrophy and Alzheimer's disease.

This thesis has studied aspects of the interaction between aluminium and the kidney. The work has addressed two major issues.

Firstly, a study is described where Malvin's stop-flow technique was used to determine any excretory/absorptive tubular site for Al in the pig kidney. Al was found to be excreted in the distal nephron of the pig kidney.

Secondly, the toxic effects of Al *in vitro* on the DNA of pig kidney cell line LLC-PK1 were investigated, in an attempt to elucidate some of the mechanisms of toxic action.

DNA synthesis was measured using ^{3}H-TdR incorporation. Over increases of both time (9-72 h) and Al concentration (0.01-8.0 mM), ^{3}H-TdR incorporation was diminished. Effects were evident at concentrations as low as 0.05 mM Al.

The production of DNA strand breaks was assessed by the increase in size of cell nucleoids (ie DNA in supercoiled form). Nucleoid size was analyzed in a Epics 753 Fluorescence Activated Cell Sorter interfaced with an MDADSII data acquisition and analysis system. After 90 min incubation with Al (over the concentration range 0.001-32 mM), an increase in nucleoid size was noted at concentrations above 0.05 mM.

The data demonstrate that Al exerts an effect on kidney cells *in vitro* which is expressed as diminished DNA synthesis and production of DNA strand breaks. These effects on DNA may have important long-term implications on various disease states associated with Al toxicity.

PREFACE

This thesis deals with aluminium excretion by the distal tubule of the pig kidney and the toxic effects of the element on cellular DNA.

The dissertation consists of three major parts:

The first part broadly reviews the literature regarding aluminium toxicology. The material from this section formed the basis of a publication which appeared in Medical Toxicology (now called Drug Safety, published by Adis Press) entitled "Monteagudo FSE, Cassidy MJD, Folb PI. Recent developments in aluminium toxicology. Medical Toxicology 1989; 4:1-16".

The second part deals with the excretion of aluminium by the kidney. The tubular handling of this process has previously been difficult to elucidate due to the unavailability of aluminium isotopes (which although they exist are extremely short lived). The technique of stop-flow analysis was used to determine the site of aluminium excretion or absorption from the pig kidney tubule. Aluminium excretion in the distal tubule of the pig kidney was demonstrated, a previously unknown finding. The substance of this work was published in Nephron (Publishers S.Karger AG, Basel) in an article entitled

"Monteagudo FSE, Isaacson LC, Wilson G, Hickman R, Folb PI. Aluminium excretion by the distal tubule of the pig kidney. Nephron 1988; 49:245-250".

The third part deals with the toxic effects of aluminium on DNA from pig kidney cells in *in vitro* culture. Using ^{3}H-TdR incorporation aluminium was shown to decrease DNA synthesis. Aluminium also caused DNA strand breaks as evidenced by increased nucleoid size on flow cytometry measurement. This work, which adds new information to the site and mechanism of toxic action of this element, was presented in part at the XIth International Congress of Pharmacology held in Amsterdam 1990, and has been recently submitted for publication.

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me through the Department of Pharmacology, University of Cape Town. Where help was received it has been acknowledged. The work has not been submitted as the requirement for any other degree.

Signed by candidate

Signature Removed

F.S.E. Monteagudo

1991

To my wife Vivienne

and our children

Julie, Sally and Luke

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INDEX

Page

SECTION B: ALUMINIUM AND RENAL EXCRETION

Page

Page

Page

Page

LIST OF ABBREVIATIONS

Al	Aluminium [alternative spelling: Aluminum]
bw	body weight
Ca	Calcium
Cr	Creatinine
EB	Ethidium bromide
FALS	Forward angle light scatter
FCM	Flow cytometer
Fr.Exc.Fil	Fractional excretion of filtered
GFAAS	Graphite furnace atomic absorption spectroscopy
GFR	Glomerular filtration rate
^{3}H-TdR	Tritiated thymidine
Mg	Magnesium
Na	Sodium
N/A	Not available - see Appendix
N/D	Not detectable - see Appendix
Pi	Phosphate
PMT	Photomultiplier tube
S	Serum
SD	Standard deviation
S-F	Stop-flow
S-F(R)	Regular stop-flow
S-F(I)	Interrupted stop-flow

LIST OF FIGURES

Page

SECTION A

REVIEW OF ALUMINIUM TOXICOLOGY

SYNOPSIS:

Aluminium is an element of increasing clinical importance (Alfrey 1981, Krueger 1984). It not only has uses as a medicinal substance but also in recent years it has been shown to be the cause of considerable toxicity, particularly in the setting of chronic renal failure. Diseases that have been shown to be associated with aluminium, or in which it has been implicated, include dialysis dementia, renal osteodystrophy and Alzheimer's disease. Aluminium also has toxicological effects on red blood cells, parathyroid hormone and chromosomes.

Aluminium is poorly absorbed from the gastrointestinal tract. Ions that are absorbed are cleared from the body mostly via the kidney. Toxicity usually occurs only when the body's physiological mechanisms are in some way undermined. The two common situations where this is found are when aluminium enters the body via a parenteral route (thus by-passing the gastrointestinal tract), and when renal function is impaired (thus reducing excretion). The latter mechanism is particularly important when combined with the excessive increase in aluminium ingestion that occurs following the use of aluminium-containing phosphate binders.

1. PHYSIOLOGY OF ALUMINIUM

Normal dietary ingestion of aluminium is approximately 3-5 mg/day, of which only about 15 µg is absorbed through the wall of the gastrointestinal tract. In humans, this small amount is usually excreted through the kidneys, maintaining the total body burden at approximately 30 mg (Alfrey 1984). Absorption of aluminium from the gastrointestinal tract has been demonstrated in humans, as well as in animal models (Ihle & Becker 1985; Kaehny *et al.* 1977b; Ott 1985; Recker *et al.* 1977; Eastwood *et al.* 1990).

Once absorbed, aluminium is bound extensively to protein, predominantly transferrin (Rahman 1984). Consequently, only about 4% of plasma aluminium is filtered at the glomerulus (Henry *et al.* 1984). Evidence exists that aluminium may be further excreted by the distal nephron, near the sodium and calcium reabsorption sites (Monteagudo *et al.* 1988). Biliary excretion of aluminium has been described, and impaired hepatic function may contribute to aluminium toxicity (Williams *et al.* 1986).

2. SOURCES OF ALUMINIUM CONTAMINATION

2.1. Contamination of water used in dialysis

Although a link has been established between dialysis encephalopathy, renal bone disease and plasma aluminium concentrations, there was initially little idea of the source of aluminium. Ward *et al.* (1978) showed a strong association between dialysis bone disease and aluminium levels in the tap water used in dialysis units in the United Kingdom. Subsequently, further epidemiological studies in Europe showed that dialysis dementia was confined to certain geographical areas. It occurred more frequently if the dialysis water was untreated or softened, it was related to the water aluminium content, and it was often associated with osteodystrophy complicated by fractures (Wing *et al.* 1980). Kaehny *et al.* (1977a) showed that significant amounts of aluminium may be transferred from dialysis fluid to the plasma during haemodialysis. Since most aluminium is then bound to a nondialyzable plasma constituent, this precludes transfer in the reverse direction. These findings have been confirmed in other studies (Fuchs *et al.* 1984; Kovalchik *et al.* 1978). Aluminium contamination of peritoneal dialysate at source has been described (Cannata *et al.* 1983). Using reverse osmosis and other

techniques the concentration of aluminium in the dialysate can currently be reduced to very low levels.

2.2. Medicines containing aluminium

Aluminium-containing antacids (such as aluminium hydroxide) are often used in the treatment of peptic ulcer disease and dyspepsia. Sucralfate, also used in the treatment of peptic ulcer disease, is an aluminium polymer which acts as a barrier-type protecting agent.

Patients with chronic renal failure are unable to excrete phosphate normally and as a result they frequently develop hyperphosphataemia. This is a major cause of secondary hyperparathyroidism which leads to renal osteodystrophy (Slatopolsky and Briker 1973). Aluminium-containing phosphate binding gels are used in these patients in order to bind dietary phosphate in the gastrointestinal tract and diminish its absorption (Andreoli *et al.* 1984). The binding gel adsorbs phosphorus through a chemical reaction to form an insoluble aluminium phosphate complex. In this way hyperphosphataemia is reduced and its adverse metabolic effects may be prevented or ameliorated. However, the use of aluminium-containing phosphate binding gels currently appears to be the major cause of aluminium toxicity in patients with chronic renal failure.

The gastrointestinal tract presents a relative barrier to ingested aluminium. It was not until the late 1970's that Kaehny *et al.* (1977b) showed that aluminium was absorbed in measurable quantities from the gastrointestinal tract of healthy volunteers. Subsequently, Andreoli *et al.* (1984) reported that aluminium intoxication developed in 3 infants with azotemia who had been treated with aluminium-containing binding gels but were not dialysed. These infants had both severe osteomalacia and raised serum aluminium concentrations, thus confirming the absorption of aluminium from the gastrointestinal tract.

2.3. Contamination of total parenteral nutrition (TPN) solutions

Patients receiving long term total parenteral nutrition have been shown to develop bone disease, as well as a number of other complications (Folb 1985). Klein *et al.* (1982) demonstrated that such patients had elevated aluminium levels in bone, urine and plasma. This finding pointed to the casein used in the total parenteral nutrition being contaminated with aluminium, and it was found that aluminium levels fell when amino acids were substituted for casein.

This was corroborated by Ott *et al.* (1983) who found that patients receiving casein in a total parenteral nutrition

solution had stainable aluminium on the surface of mineralised bone in a pattern similar to that found in dialysis osteomalacia. The same pattern was not found in patients receiving parenteral nutrition with amino acids as their nitrogen source.

The American Food and Drug Administration (FDA) has considered imposing limits on the aluminium content of parenteral drug products (less than 100 µg/day). The FDA considers three groups of patients to be at particular risk, viz patients with renal failure, patients receiving long-term parenteral nutrition, and premature infants (WHO Advisory Notice 1990).

2.4. Contamination of human serum albumin

Plasmapheresis (plasma exchange) is a clinical procedure in which plasma is exchanged for a solution containing electrolytes and human serum albumin. In this way harmful substances in the plasma, such as immune complexes or lipoproteins can be removed.

Recent work has demonstrated that in the process of its purification albumin may become heavily contaminated with aluminium. This was first described by Milliner *et al.* (1985), who showed that plasma and urine aluminium rose in a patient after plasma exchange. They found that this aluminium loading was due to contamination of the

albumin, probably during manufacture (Loeliger & de Wolff 1985). This was subsequently confirmed in 4 other patients (Monteagudo *et al.* 1987).

Plasmapheresis has been used in the past in an attempt to reduce the aluminium loading in patients with chronic renal failure (Elliot *et al.* 1978b). Although the body burden was apparently reduced, no long term benefit from this procedure was observed. This technique is unlikely to be of value, and is not recommended for the treatment of aluminium toxicity.

A recent case report described a marked rise in plasma aluminium in a woman receiving human serum albumin for hypoalbuminaemia during continuous ambulatory peritoneal dialysis (Maher *et al.* 1986). The plasma aluminium level fell on discontinuation of the albumin. This problem is likely to continue to manifest itself in other situations where albumin is used, although the clinical significance has been questioned (Fell *et al.* 1986).

2.5. Contamination of fluids used in infants

Sedman *et al.* (1985) recently reported raised plasma, urinary and bone aluminium concentrations in premature infants compared with controls, and postulated that this group is at special risk because of the likelihood of

parenteral exposure and poor renal clearance. Many of the intravenous solutions investigated by these workers (in particular potassium phosphate, sodium phosphate, calcium gluconate and serum albumin) were found to be heavily contaminated with aluminium. The same findings were not confirmed, however, in a group of full term infants, emphasising the possible role of impaired renal function in premature neonates (Puntis *et al.* 1986).

Freundlich *et al.* (1985) reported the findings of raised brain aluminium in postmortem specimens from two neonates with uraemia. Fluids to which these patients were exposed were analysed and found to contain negligible amounts of aluminium. However, the infant milk formula used ('Similac PM 60/40') had an elevated aluminium concentration and was thought to be the most likely source of contamination.

Concern has also been expressed about the use of aluminium-containing injected vaccines in infants (Lione 1986).

2.6. Contamination from environmental and industrial sources

Aluminium has been implicated in neurological disorders (incoordination, intention tremor and some cognitive defects) found in aluminium smelter workers (Longstreth

et al. 1985). In addition, workers in aluminium smelters may have a greater incidence of bladder carcinoma than the general population (Theriault *et al.* 1984), but no other risk of mutagenicity has been demonstrated in humans. No reports on the teratogenicity of aluminium are available.

Atmospheric pollution in terms of sulphuric and nitric acid (so-called acid rain) has increased the leaching of aluminium from the soil. This has resulted in the elevation of aluminium concentration in the surface and ground water, where it has been shown to accumulate in the food chain (Cronan & Schofield 1979; Cronan *et al.* 1978, 1986; Nordstrom & Ball 1986). Indeed, aluminium is an important participant in the neutralisation of strong acids in the soil (Johnson 1979). Thus, in an insidious way the effect of acid rain on aluminium solubility in the soil and the subsequent leaching of the element into nearby bodies of water may have important ecological consequences. Other writers have expressed concern about the use of aluminium cooking pots (Levick 1980), and about the levels of aluminium in various beverages such as tea (Coriat & Gillard 1986; Koch *et al.* 1988).

3. MEASUREMENT OF ALUMINIUM

The most popular and accurate method of aluminium measurement is by graphite furnace atomic absorption

spectroscopy (D'Haese *et al.* 1985; Parkinson *et al.* 1982). Measurements by this technique give values in the parts per billion (ppb) range and are often expressed as µg/L or µmol/L. Normal human plasma aluminium values are generally accepted to be less than 10 µg/L, although values ranging from 2,1 to 42 µg/L have been described. Major problems in the assay technique at this level of sensitivity include that of contamination by the environment, in particular by metals (e.g. from needles), glassware (e.g. laboratory containers), analytical reagents (including water) and airborne dust (Frech *et al.* 1982). Controversies still exist about the accuracy of aluminium assays in biological samples, particularly in serum (Adan *et al.* 1985; Berlyne & Adler 1985; Burnatowska-Hledin & Mayor 1985; Cornelis & Schutyser 1984).

Other methods of detecting aluminium have included histochemical techniques that stain the element (Pearse 1972) and neutron activation analysis (Turkstra *et al.* 1978).

No stable radioactive isotopes of aluminium exist, and as a result no tracer studies using isotopes have been possible. This has inhibited aluminium research considerably, as a number of current investigative techniques are dependent on the existence of isotopes.

4. EXPERIMENTAL STUDIES

4.1. Experimental studies in animals

One of the earliest animal studies involving aluminium was conducted by Berlyne and co-workers (1972). They demonstrated a syndrome associated with periorbital bleeding, lethargy, anorexia and death due to aluminium loading in uraemic and non-uraemic rats, with the former being more seriously affected. Plasma and tissue concentrations of aluminium were found to be elevated. This experiment demonstrated the important effect of renal excretion on aluminium clearance by the body and the resultant toxicity in uraemic animals.

Henry *et al.* (1984) administered 1 mg/kg of aluminium intravenously to dogs, and measured various parameters. The plasma half-life of the element was calculated to be 276 $\pm$ 51.8 minutes, and the volume of distribution 1.30 $\pm$ 0.17 liters or 5.90 $\pm$ 0.30% of body weight. 10 to 21% of administered aluminium was found to be excreted in the urine over 150 min. The renal contribution to plasma clearance of aluminium correlated with the glomerular filtration rate (GFR). The total plasma clearance of aluminium (0.27 $\pm$ 0.17 L/h) was greater than the renal aluminium clearance (0.12 $\pm$ 0.02 L/h), thus confirming the widely held view that aluminium is mainly, but not entirely, excreted renally. The difference between total

plasma clearance and renal clearance may be accounted for by the distribution of the aluminium into other body compartments such as bone, liver and brain.

Henry *et al.* (1984) confirmed that aluminium is largely protein-bound. When measuring plasma concentrations, both protein-bound and free aluminium are measured. However, since proteins are normally not filtered by the glomeruli, the aluminium appearing in the urine presumably derives from the free fraction in the plasma, provided none is secreted or absorbed. In an effort to quantify the free aluminium fraction in plasma, Henry *et al.* (1984) examined the ultrafilterable fraction of aluminium *in vivo* (during a haemodialysis procedure in a dog) and found it to be 3.9%. They also looked at *in vitro* ultrafiltration by centrifuging plasma through a membrane and found the diffusable fraction to be 2.5%. Although these figures suggest that only a small fraction is filtered by the glomerulus, no data exist to show the true glomerular filtered fraction. Henry *et al.* (1984) measured total plasma levels of aluminium, and therefore the renal clearance of aluminium as measured by them does not have the same meaning as the "usual" renal clearance.

A study of the renal excretion of aluminium in the rat using micropuncture techniques (Burnatowska-Hledin *et al.* 1985) confirmed previous work (Henry *et al.* 1984)

showing that less than 8.4% of plasma aluminium is ultrafilterable.

4.2. Human studies

Recker *et al.* (1977) demonstrated that aluminium may be absorbed in considerable amounts if administered orally. They gave a group of 6 volunteers aluminium carbonate orally and monitored the urinary concentrations of aluminium before and after the loading. The daily urinary aluminium excretion in the two control days was found to be 85.8 $\pm$ 64.9 µg while during the 4 days of aluminium loading the daily urine Al ranged from non-detectable to 1147 µg with an average steady-state urinary aluminium excretion of 495µg/d.

Other available data are from clinical work and refer to aluminium measurements in the setting of a particular medical problem. Kaehny *et al.* (1977a) described two groups of patients with chronic renal failure, one dialysed with high-aluminium dialysate, the other dialysed with low-aluminium dialysate. They found that the low aluminium group excreted 9 $\pm$ 9 µg/d and the high aluminium group 48 $\pm$ 19 µg/d in the urine. The high-aluminium group were then dialysed for a period of 2 months with low-aluminium-dialysate and their urinary excretion fell to 19 $\pm$ 9 µg/day.

In their observation of inadvertent aluminium administration during a single plasma exchange, Milliner *et al.* (1985) found that aluminium concentrations in the urine increased. The levels were highest on the first day and fell over the following two days. It is of interest that although serum aluminium concentrations returned to baseline within 24 hours of the exchange, urinary aluminium levels remained elevated for a further 48 hours, implying sequestration and subsequent mobilisation of aluminium from other body compartments.

5. PATHOPHYSIOLOGY

5.1. Aluminium, bone disease and the parathyroid gland

Osteitis fibrosa cystica is a virtually universal accompaniment of chronic renal failure; osteomalacia is less common (Ellis & Peart 1973). The incidence of osteomalacia in patients prior to dialysis has varied according to the diagnostic criteria used, from 33% in Newcastle (Mora Palma *et al.* 1983) to 78% in Kentucky (Malluche *et al.* 1976). However, a severe form of fracturing osteodystrophy which histologically was due to osteomalacia was a major problem in certain dialysis units during the 1960s and 1970s (Pierides *et al.* 1980). Ionic aluminium readily crosses a dialysis membrane, and a patient undergoing haemodialysis is exposed to between 300 to 400 litres of water each week across this

membrane. Dialysis units with a water supply that was high in aluminium experienced the greatest problems (Parkinson 1979). The incidence of this form of osteomalacia was clearly related to the duration of dialysis and was causally related to aluminium.

The incidence of aluminium-related bone disease and the risk factors associated with its development since the advent of water treatment are difficult to determine. Malluche (1987) found stainable aluminium in 49% of 150 unselected dialysis patients at his institution. In addition, 5% of predialysis patients with a glomerular filtration rate of between 10 and 15 ml/min had stainable bone aluminium. In a retrospective study from Montreal, chronic dialysis patients exposed to water with an aluminium content of less than 10 µg/L, 25% of patients undergoing bone biopsy had aluminium-related osteomalacia (Turner *et al.* 1988).

Aluminium localisation in bone is demonstrable at the mineralisation front in trabecular bone using aurine stain. The rate of bone formation is inversely related to the amount of aluminium present (Ott *et al.* 1982), and can be evaluated by the incorporation of tetracycline into the mineralisation front. By using two time-spaced doses of oral tetracycline the rate of mineralisation can be quantitatively determined by microscopic examination

of the bone and measuring the distance between the two tetracycline labels.

The mechanism by which aluminium causes osteomalacia is not fully established, and it may be multifactorial. Aluminium has been shown directly to inhibit osteoblast function (Dunstan *et al.* 1984), and deposits of aluminium within the mitochondria of osteoblasts in patients with aluminium bone disease can be detected on electron microscopy (Cournot-Witmer *et al.* 1986). The normal osteoblastic response to parathyroid hormone is reduced in the presence of aluminium. Furthermore, aluminium acts extracellularly at the mineralisation front, inhibiting the formation and aggregation of new bone crystals (Posner *et al.* 1986).

The effect of aluminium on bone is mainly seen in trabecular bone. The difference in effect between trabecular and cortical bone may simply reflect the fact that the former is metabolically more active than the latter, or it may be due to local tissue factors. Certain patient groups appear more susceptible to aluminium deposition, for example the deposition of aluminium on bone surfaces in diabetic uraemic patients is enhanced compared with normals (Andress *et al.* 1987).

The natural course of aluminium osteomalacia if the aluminium source is not removed is one of progressive

bone pain, fractures and deformity which are crippling (Ward *et al.* 1978). Bone pain may be an early feature, often occurring first in the feet and back (on exercise or standing), before becoming more generalised. In addition, a proximal myopathy occurs in the majority of patients, further aggravating the limitation of movement caused by bone pain. These clinical features are quite distinct from those found in bone disease associated with secondary hyperparathyroidism (osteitis fibrosa cystica) where bone pain is not a significant feature even in advanced cases.

Laboratory and radiological features of the two conditions are also quite distinct. Patients with aluminium related bone disease typically have normal serum alkaline phosphatase, normal serum calcium, normal or high serum phosphate, and low circulating parathyroid hormone. Incorporation of calcium into bone in the presence of aluminium is impaired, and hypercalcaemia may readily occur in this setting with the administration of Vitamin D. Radiological hallmarks of the disease are osteopaenia, pathological fractures and Looser's zones although these features are usually only seen in advanced cases. In contrast, patients with osteitis fibrosa cystica characteristically have high serum alkaline phosphatase, low or normal serum calcium, high serum phosphate, high circulating parathyroid hormone,

radiological evidence of subperiosteal erosions, and a good response to Vitamin D (Massry 1986).

Parathyroid hormone has been implicated in the pathogenesis of aluminium-related renal bone disease as well as in altering the distribution of aluminium in tissues (Alfrey 1985b; Mayor 1985). The role of parathyroid hormone and phosphate in bone disease is well known, and it may be that it is partly through these mechanisms that some of the effects of aluminium on bone are mediated (Kaye & Gagnon 1985). Although aluminium binds dietary phosphate due to its physicochemical properties, it is possible that it may exert additional, less well-defined effects on phosphate metabolism. Urinary phosphate has been shown to decrease after aluminium loading in both animal and human studies (Ondreicka *et al.* 1966; Recker *et al.* 1977).

Henry *et al.* (1984) showed that 150 minutes after a single injection of aluminium the parathyroid hormone levels in dogs fell by 27 $\pm$ 4%. They noted that although the calcium in the serum and the urine rose 15 minutes after injection this was unlikely to be a hormonal effect because of the rapidity of the response. Moreover, ionised calcium, which is thought to be the calcium fraction causing a parathyroid hormone response, did not increase. The serum phosphate rose slightly during the 150 minutes, but this rise was significantly different

only at 30 minutes compared with baseline. For reasons that were not explained urinary phosphate concentrations were not studied.

Mayor *et al.* (1977, 1980) studied rats that had been loaded with oral aluminium, and noted that parathyroid hormone increased the absorption of aluminium by the gastrointestinal tract. Levels of aluminium in various tissues also increased significantly. The authors confirmed these findings by withdrawing parathyroid hormone administration and noting the resultant fall in tissue aluminium levels. This effect might be influenced by 1,25-dihydroxy cholecalciferol (Burnatowska-Hledin *et al.* 1984).

Cann *et al.* (1979) studied aluminium levels in parathyroid glands, thyroid and cervical muscle in healthy and hyperparathyroid human subjects and in rats. They found that in all these categories the level of aluminium in the parathyroid glands was significantly greater than in other tissues, and was linearly related to dietary aluminium intake.

Morrissey *et al.* (1983) found that by increasing the quantity of aluminium from 0.5 to 2.0 mmol/L in an *in vitro* parathyroid cell culture they inhibited the secretion of parathyroid hormone. This effect was reversible, as normal secretion returned on placing the

cells in an aluminium-free medium. Using radiolabelling techniques they demonstrated that the inhibitory effect was due to decreased secretion of parathyroid hormone from the cell rather than interference with biosynthesis within the cell.

The inhibitory effect of aluminium on bone may in part be brought about by its effect on the parathyroid gland. Parathyroid hormone secretion is regulated by protein kinase C (Morrissey 1984), which in turn is dependent on calcium, phospholipids and diglyceride levels. Aluminium has been shown to reduce diglyceride levels in dispersed bovine parathyroid cells and thereby reduce parathyroid hormone secretion (Morrissey & Slatopolsky 1986). Parathyroidectomy in patients with chronic renal failure is associated with enhanced bone deposition of aluminium (Andress *et al.* 1985). It has been shown that aluminium-induced osteomalacia may deteriorate after parathyroidectomy in some patients (Felsenfeld *et al.* 1982).

Parathyroid hormone has been shown to modulate toxicity of other metals such as cadmium (Washko & Cousins 1979) and lead (Six & Goyer 1970), in addition to aluminium.

5.2. Aluminium and the brain

The first reported case of human aluminium intoxication presented with neurological manifestations, namely memory loss, tremor, jerking movements and impaired coordination (Spofforth 1921). Since then aluminium has been associated with several neurological syndromes.

5.2.1. Alzheimer's disease

Alzheimer's disease is a dementing illness that afflicts approximately 2 million Americans, and it has been estimated that at least 100 000 of them will die from the disease every year (Wurtman 1985). Various hypotheses about the aetiology of this disease exist, of which the aluminium theory is but one (Crapper *et al.* 1973, 1976; Delaney 1979; Foncin 1987; Perl & Brody 1980; Shore *et al.* 1980; Wascher & Cohn 1985; Yates 1980). The nuclei of brain cells containing neurofibrillary tangles (which are the distinctive histological feature of Alzheimer's disease) have been shown to have nuclei which are, in a high percentage of cases, positive for aluminium when studied by scanning electron microscopy and x-ray spectrometry (Perl & Brody 1980). Injection of aluminium into the brains of certain animals has induced the development of similar neurofibrillary tangles (Crapper *et al.* 1973). Aluminium has been shown to affect the blood-brain barrier by increasing its permeability, and altering membrane function (Banks & Kastin 1983; Banks & Kastin 1989).

A controversial epidemiological study of 88 county districts in England and Wales showed that the risk of Alzheimer's disease was 1.5 times higher in districts with an aluminium concentration in the drinking water greater than 0.11 mg/L than in districts with water aluminium levels smaller than 0.01 mg/L (Martyn *et al.* 1989).

5.2.2. **Dialysis encephalopathy**

It has been suggested that aluminium might play a role in the pathogenesis of the dialysis encephalopathy syndrome (Alfrey *et al.* 1976) and raised aluminium levels have been found in post-mortem brain specimens (McDermott *et al.* 1978). That aluminium is the cause of dialysis dementia is now beyond doubt (Alfrey 1985a, Arieff 1985). Non-specific symptoms often precede the condition including malaise, loss of memory and concentration, depression and anxiety. The clinical features are a progression of stuttering to aphasia; twitching to myoclonus; and mild personality disorders to seizures and dementia. Typical electroencephalographic abnormalities include generalised slowing of the normal rhythm with bursts of high voltage, slow frequency waves. These features are characteristic, but not diagnostic, of dialysis dementia (Smith *et al.* 1978).

How aluminium exerts its toxicity on the brain in this condition is unclear. Using histological techniques aluminium has been shown to concentrate predominantly in the grey matter of the brain. This histological hallmark of toxicity in experimental animals is neurofibrillary tangles (Wisniewski *et al.* 1970), which are similar but not identical to those found in Alzheimer's disease (Crapper *et al.* 1973). In patients dying of dialysis dementia, however, the pathological changes in the brain are usually nonspecific and neurofibrillary tangles are rarely found (Bugiani *et al.* 1985).

5.2.3 **Other disorders**

In addition to Alzheimer's disease and dialysis dementia, aluminium has been associated with other neurological disorders such as amyotrophic lateral sclerosis and Parkinsonism- dementia found in the islands of Guam (Perl *et al.* 1982). Recent work has also indicated that aluminium overload may also predispose to epileptic seizures in renal transplant patients treated with cyclosporin (Nordal *et al.* 1985).

5.2.4 **Biochemical considerations**

At the molecular level, aluminium may affect brain biochemistry by various mechanisms. Aluminium has been shown to affect synaptosomal membranes *in vitro* (Lai *et*

al. 1980), and to decrease synaptosomal uptake of glutamic acid, gamma-aminobutyric acid (GABA) and glycine (Wong *et al.* 1981). It is also possible that aluminium interferes with brain cytochrome oxidase activity. Animal studies have shown that after aluminium ingestion the levels of adenosine triphosphate (ATP) in the blood fall, and the levels of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) rise (Ondreicka *et al.* 1966). These changes were accompanied by impaired incorporation of RNA and DNA into the tissues (Ondreicka *et al.* 1966). Within 6 hours of intrathecal aluminium injection the element can be shown histochemically to be preferentially attached to nuclear chromatin in cerebral cortical cells (De Boni *et al.* 1974). In animal studies aluminium-induced neurofibrillary tangles in the central nervous system have been associated with an increase in brain aluminium and a decrease in the activity of choline acetyl transferase and acetyl cholinesterase (Yates *et al.* 1980). More recently it has been found (Altmann *et al.* 1987) that aluminium may inhibit dihydropteridine reductase (DHPR) activity in erythrocytes. This enzyme is essential for the normal synthesis of a specific neurotransmitter, in addition to being found in erythrocytes. Although it has not been measured in the brains of dialysis patients it may be that erythrocyte levels of DHPR reflect brain levels.

5.3. Aluminium and anaemia

Aluminium rapidly distributes between plasma and red blood cells, with only small quantitative differences existing between the two compartments (van der Voet & de Wolff 1985).

It was demonstrated as long ago as 1929 that aluminium toxicity in animals results in an anaemia similar to that found in lead poisoning (Siebert & Wells 1929). In humans the observation was made in 1978 (Elliot & MacDougall 1978a) that some dialysis patients with encephalopathy and some with aluminium related bone disease develops a severe hypochromic microcytic anaemia despite adequate iron treatment. O'Hare and Murnaghan (1982) demonstrated that there was an improvement in the anaemia of 15 chronic haemodialysis patients who were transferred from a high-aluminium to a low-aluminium water supply. This was confirmed by others (Touam *et al.* 1983). Studies in humans thus far only provide circumstantial evidence that aluminium toxicity produces anaemia, but the evidence of animal studies is less equivocal (Berlyne *et al.* 1972).

There are several mechanisms that may be implicated in the pathogenesis of the anaemia. Various metals, including aluminium, affect the activity of delta-aminolaevulinic acid (d-ALA) dehydratase (McGonigle

& Parsons 1985). However, clinical studies show a wide scatter in the activity of d-ALA hydratase which does not correlate with blood aluminium levels. Aluminium in blood is bound to transferrin (Rahman *et al.* 1984), and it is possible that by preventing loading or unloading of iron onto this molecule anaemia may be produced. Interference with iron transport may also occur in the bone marrow. Aluminium is deposited in macrophages within the marrow and this may interfere with the macrophages' role of digesting redundant erythrocytes and recycling iron. The effects of aluminium on DNA (Karlik *et al.* 1986) and calmodulin (Siegel & Haug 1983) *in vitro* suggest other hypotheses for the molecular basis of the anaemia.

6. TREATMENT

The management of aluminium toxicity can be classified into three major areas: the identification of the source of the aluminium, the removal of the aluminium from that source, and the use of desferrioxamine.

6.1. Identification of aluminium sources

This has been dealt with in a previous section, which described some of the currently known sources of aluminium. It will be important in the future to

maintain a high degree of suspicion in detecting new sources of aluminium loading.

6.2. Removal of aluminium

6.2.1. Removal from water

Patients with renal failure undergoing haemodialysis have been identified as a group at risk from long term aluminium accumulation, due to the contamination of the water used for dialysis. The concentration of aluminium in municipal water supplies varies from place to place, and this explains the clustering of dialysis dementia and bone disease in certain renal units (Barratt & Lawrence 1975; Hudson *et al.* 1979; Wing *et al.* 1980). This variation in aluminium content may be due to the fact that in many areas municipal water is treated by alum precipitation. Reports from different centres throughout the world have since confirmed the beneficial effects of removing aluminium from water used for dialysis (Hodge *et al.* 1981; Kerr *et al.* 1986; Milne *et al.* 1982; Platts & Anastassiades 1981).

There is no "safe" level of water contamination with aluminium, although it has been suggested that at dialysate aluminium concentrations greater than 14 µg/L aluminium transfer will occur (Hodge *et al.* 1981). One epidemiological study showed that few cases of fracturing

osteodystrophy occurred in centres where the local tap water contained less than 50 µg/L (Parkinson *et al.* 1979). It has, however, been shown that aluminium accumulation can occur in a few long-term haemodialysis patients treated with a dialysate containing less than 30 µg/L of the element (although these patients were also prescribed aluminium-containing phosphate binding gels) (Kerr *et al.* 1986).

The two usual methods for water purification in dialysis units are deionisation and reverse osmosis. Deionisers are used for the removal of dissolved inorganic ions; they contain cationic resins which exchange hydrogen ions for dissolved cations and anionic resins which exchange hydroxyl groups for dissolved anions.

Potential problems with deioniser use are bacterial contamination and an acidic effluent as the exchange capacity of the deioniser is saturated. The latter can lead to leaching of copper from pipes and copper toxicity (Manzler & Schreiner 1970). It may also inactivate heparin, leading to clotting of dialysers (Schwarzbeck *et al.* 1977).

Reverse osmosis is superior to deionisation in the removal of aluminium. In addition to removing dissolved inorganic ions it removes dissolved organic substances, particulate matter, bacteria and pyrogens. The principle

of the technique is based on molecular sieving and ionic exclusion. Pressure is applied across a membrane (of which there are several types eg. cellulose). When the applied pressure is greater than the osmotic pressure solvent will move from the more concentrated side of the membrane to the other.

6.2.2 **Removal from phosphate binders**

Aluminium-containing phosphate binding gels are often important therapy for patients with chronic renal failure and associated high serum phosphate levels. They are used to bind dietary phosphate and thus decrease its absorption. However, it has been shown by Kaehny *et al.* (1977b) that aluminium is absorbed from aluminium-containing phosphate binding gels in the gastrointestinal tract. Consequently, nephrologists have become cautious about their use. An important practical point is that the binding gels should only be given at the time of main meals, to provide optimal phosphate binding. Phosphate control appears to be better in those patients who have normal gastric acid secretion (Cassidy *et al.* 1988) and the capacity of aluminium-containing phosphate binding gels to bind phosphate has been shown *in vitro* to be closely related to pH (Balasa *et al.* 1987).

Calcium carbonate has been demonstrated to be an effective phosphate binder both in adults (Slatopolsky *et al.* 1986) and in children (Andreoli *et al.* 1987) with chronic renal failure. Other centres have been using magnesium-containing antacids in combination with magnesium-free dialysate in similar attempts to minimise aluminium exposure (Wheeler *et al.* 1986). Some workers have, however, found this to be ineffective and associated with magnesium toxicity (Jennings *et al.* 1986). Other than calcium carbonate, no satisfactory non-toxic phosphate binders are currently available. More recently, natural polymeric anions have been investigated as phosphate binders, with encouraging results (Schneider *et al.* 1983). Effective non-aluminium containing phosphate binders which are also non-toxic would be a major development in nephrology. Current practice is to recommend the use of calcium carbonate together with reduced amounts of aluminium-containing phosphate binding gels when necessary.

6.2.3. **Desferrioxamine**

Desferrioxamine is principally used in practice to chelate iron. It also chelates various other metal ions, and its affinity for aluminium is high (Swartz 1985). The aluminium-desferrioxamine complex has a molecular weight of approximately 587 daltons (Leung *et al.* 1985)

and is dialysable. As it is not well absorbed orally desferrioxamine must be administered parenterally for systemic effect. It is predominantly excreted by the kidney, with more than 70% of an administered dose appearing in the urine of patients with normal renal function. Ackrill and colleagues were the first to report its use intravenously in the treatment of aluminium overload in chronic haemodialysis patients (Ackrill *et al.* 1980).

There have been several subsequent reports on the effectiveness of desferrioxamine in reversing the manifestations of aluminium toxicity in dialysis dementia (Ackrill *et al.* 1986; Milne *et al.* 1983; Pogglitsch *et al.* 1981), aluminium related anaemia (Swartz *et al.* 1985), and aluminium related osteodystrophy (Ackrill *et al.* 1982; Ihle *et al.* 1982; Malluche *et al.* 1984). Desferrioxamine can be given intravenously, subcutaneously or intraperitoneally (for those patients on chronic ambulatory peritoneal dialysis). The best mode of administration and the optimum dose have not yet been established. Smaller doses given more frequently (1 gram three times weekly) (Ihle *et al.* 1982) have been shown to be as effective as larger doses in removing aluminium. Malluche *et al.* (1984) used a dose of 28.5mg/kg 3 times weekly infused during the first two hours of dialysis. With this dose no complications were seen during a 10-month follow-up period. Infusion of

desferrioxamine at a dose of between 15 and 20mg/kg body weight once weekly, during the last hour of dialysis is also practised. Administration at the end of dialysis rather than at the beginning minimises loss of desferrioxamine in the dialysate.

Data on whether the type of dialysis membrane used affects the aluminium-desferrioxamine complex clearance are conflicting. While some authors have demonstrated that high flux membranes (e.g. polyacrilonitrile) have been associated with high clearances compared with standard cuprophane membranes (Chang & Barre 1983), others have not shown this association (Bonal *et al.* 1984; Malberti *et al.* 1986; Muirhead *et al.* 1986). It would, however, be expected in view of the aluminium-desferrioxamine complex, that clearance would be more efficient with high flux dialysis membranes.

The necessary duration of treatment with desferrioxamine is also unclear. A striking clinical improvement is seen in patients with proximal myopathy and bone pain, often within 1 month of starting therapy (Malluche *et al.* 1984). Interestingly, this clinical improvement is seen before a large amount of aluminium can have been removed. It would seem that treatment is required for 6 to 9 months in many cases.

A single infusion of desferrioxamine results in peak serum levels of Al 24 to 40 hours after administration, and this can be used to determine the body load of aluminium (Milliner *et al.* 1984). Malluche *et al.* (1984) found that 12 patients with aluminium accumulation in bone experienced a rise in serum aluminium following a single desferrioxamine infusion, but elevations were also seen in some of 10 patients without bone aluminium accumulation. The investigators concluded that the test cannot be used specifically to diagnose the accumulation of aluminium in bone, and suggested that the rise in serum aluminium seen in patients without bone accumulation may have been due to release of the metal from other tissues.

Another explanation may be contamination of the desferrioxamine itself with aluminium. This has been demonstrated recently in three batches of commercially available desferrioxamine (Wagner *et al.* 1985). The desferrioxamine test is not absolutely specific or sensitive and a reasonable guideline would be to consider an increment in the serum aluminium of 150 µg/L or more as indicating a positive test (de Broe *et al.* 1988).

Iron deficiency due to depletion of total body stores may occur during desferrioxamine treatment, although this is rare. However, serum ferritin levels fall significantly

with treatment (Swartz *et al.* 1985). Although other trace metals such as manganese, copper, tin and zinc bind to desferrioxamine, deficiency states have not been reported on treatment. Bone copper has been shown to be depleted during treatment with desferrioxamine but a true deficiency state has not been reported (Hewitt *et al.* 1986).

Other more serious side effects of desferrioxamine treatment include anaphylactic reactions and visual disturbances (Davies *et al.* 1983; Simon *et al.* 1983). In addition, there have now been several reports of serious and fatal cases of opportunistic infections such as mucormycosis (Eiser *et al.* 1987; Windus *et al.* 1987) and systemic yersiniosis (Boyce *et al.* 1985; Robins-Browne & Prpic 1983) in patients receiving desferrioxamine. Many of these side effects are probably dose related.

Desferrioxamine scavenges free radicals and modifies neutrophil and macrophage function (Halliwell & Gutteridge 1985), which may affect host immunity. It has been shown that the virulence of yersinia is increased by the presence of desferrioxamine (Robins-Browne & Prpic 1983). Whether or not it can be utilised by the Mucorales fungi to affect their growth in a similar fashion is not known. In addition, desferrioxamine may inhibit the fungistatic properties of transferrin (Holzberg & Artis 1983). It is currently unclear which

of these mechanisms, if any, are important in the pathogenesis of this opportunistic infection, and if there is a sub-population of dialysis patients specifically at risk. Occasionally, transient worsening of dialysis dementia has been reported, in association with the short-lived rise in plasma aluminium levels that may occur after desferrioxamine administration (Ackrill *et al.* 1980).

7. CONCLUSION

There has been an explosion of knowledge with regard to aluminium in the last decade. Aluminium has been shown to be involved in pathological processes involving primarily brain and bone, particularly in patients with renal failure. In addition, the numerous ways a person can be exposed to aluminium (mainly iatrogenic) have become better known. However, many of the public health concerns about the toxicity of this substance in the ill, as well as the healthy population, remain unanswered at present.

SECTION B

ALUMINIUM AND RENAL EXCRETION

SYNOPSIS:

It is known that the excretion and absorption of various ions takes place at defined sites along renal tubules. Although it is known that the kidney as a whole excretes Al, the mechanisms by which this takes place are largely unknown.

In this section a study is described where Malvin's stop-flow technique was used to determine any excretory/absorptive tubular site for Al.

Al was found to be excreted in the distal nephron of the pig kidney.

1. INTRODUCTION

1.1 Renal handling of aluminium

Al is excreted primarily by the kidneys (Alfrey *et al.* 1976). Various kinetic studies have been performed on Al renal excretion (Henry *et al.* 1984; Hohr *et al.* 1989). Very few studies have addressed the issue of the renal tubular handling of this element. Where along the tubule, if at all, excretion or absorption takes place is not definitively established.

The reasons for this lack of knowledge may relate to the very small quantities of sample volume produced by techniques such as micropuncture and isolated perfused tubules (Burg 1982). No stable isotope of aluminium exists which might make the assay of the element in nanolitre volumes feasible. Using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) the volume of sample required is in the order of microlitres (D'Haese *et al.* 1985; Parkinson *et al.* 1982). The use of micropuncture or perfused isolated tubule techniques producing nanolitre volumes is therefore not possible in the direct study of aluminium transport in the renal tubule.

Burnatowska-Hledin *et al.* (1985) noted the problem of the nanolitre quantities produced by micropuncture

techniques, stating in their paper "However, a direct determination of aluminum concentration in tubular fluid was not feasible because of the sample size required for analysis with presently available methods". Their data indicated that at elevated plasma aluminium levels less than 8.4% of plasma aluminium was ultra-filterable suggesting extensive plasma protein binding. Increased excretion of aluminium in response to volume expansion was noted but not to furosemide. The authors thus suggested the proximal tubule as a major site of aluminium reabsorption. This observation was made by inference only, as the authors themselves acknowledged in their paper (Burnatowska-Hledin *et al.* 1985).

Review of the literature has not produced evidence of other work dealing with the tubular handling of Al.

1.2 Introduction to stop-flow experiments

1.2.1 Alternative techniques

Renal tubular transport mechanisms are routinely studied using kidney micropuncture or perfusion of isolated tubule segments (Burnatowska-Hledin *et al.* 1985; Stokes 1982; Burg *et al.* 1966; Burg 1982). A major drawback of both these techniques is that they produce nanolitre volumes of tubular fluid for analysis.

The only technique allowing the direct study of renal tubular handling of Al (because it produces substantial assay volumes suitable for GFAAS assay) is the stop-flow technique described by Malvin *et al.* (1958a; 1958b). This technique permits localization of sites of maximal absorption or excretion of various substances along the length of the nephron.

1.2.2. **Concept of stop-flow**

Malvin *et al.* explained the concept of their technique in a publication (1958a). The following is a quotation from the article describing the concept:

> *"The method depends upon the concentration pattern derived from urine samples caught serially from a catheter following a brief period of ureteral occlusion. If a dog is infused with a high load of osmotic diuretic, such as mannitol, the urine to plasma concentration ratios (U/P) for most substances approach 1.0. If now the ureteral catheter is occluded, filtration at the glomeruli will stop when the tubular pressure becomes equal to the net filtration pressure. The urine in the tubules which had been relatively unchanged from that of glomerular filtrate will now be exposed to the action of the various nephron segments for as long as the ureteral occlusion lasts. Previous*

administration of the osmotic diuretic provides a watery menstruum against which electrolytes may be reabsorbed. The various substances will now be reabsorbed or secreted more completely, and new U/P ratios developed which will differ from the control U/P ratio. Also, the concentration of any substance will vary along the nephron depending upon how the individual segments handle that substance. Creatinine concentrations might be expected to vary along the entire nephron only as a result of water movement, secreted substances would show an increase in concentration in the nephron areas which secrete that substance, while the concentration of reabsorbable solutes would fall in those areas responsible for reabsorption. After ample time has been allowed for the concentration patterns to develop during stopped flow the ureteral occlusion is released. Rapid serial urine samples are taken from the new flow. The concentration pattern is caught in these samples collected rapid fire from the polyethylene ureteral catheter after the brief occlusion is released."

Figure 1 illustrates the concept involved.

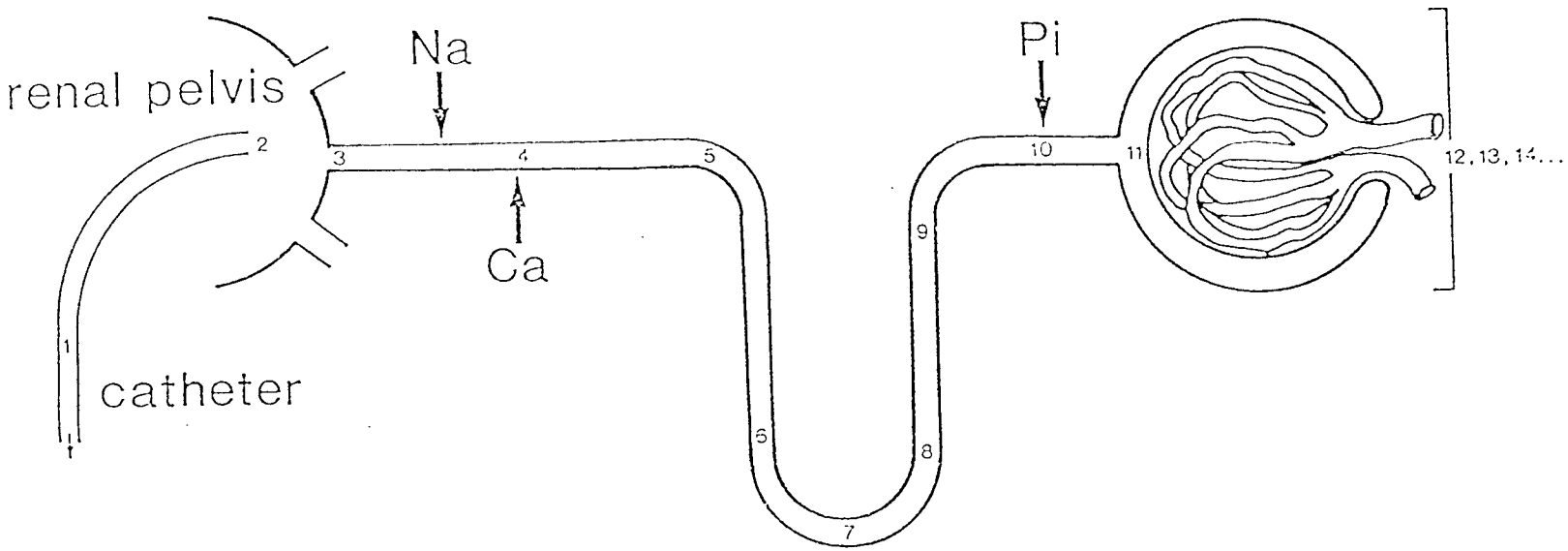

FIGURE 1: Diagram demonstrating the concept of stop-flow. The sodium (Na), calcium (Ca) and phosphate (Pi) sites of absorption against their concentration gradients are marked.

1.2.3. Rationale for the interupted stop-flow technique

The interrupted stop-flow technique was described by Murdaugh and Robinson (1960). It is a modification of Malvin's stop-flow technique and consists of two consecutive ureteral occlusions separated by a brief period during which the first ureteral occlusion is temporarily relieved and the released fluid discarded. Subsequent to the second occlusion the urine samples are serially collected in the usual stop-flow fashion.

By using this technique the proximal tubular fluid modified during the first period of stop flow will move downstream into the distal nephron during the period of temporary release following the first occlusion. This facilitates the demonstration of distal excretion of those solutes which had been reduced to low concentrations proximally.

1.2.4. Critique of the stop flow method

The crucial advantage of the stop flow method is that it produces large sample volumes which allow for the assay of aluminium by GFAAS.

Drawbacks of the technique include the fact that the samples collected are not coming from a single tubule but collectively from all the tubules in the kidney. These

tubules are obviously of varying lengths, thus when we discuss a "tubule" in the stop-flow context it is a hypothetical structure. The localization of any reabsorption or excretion site to a limited precise area of the tubule is therefore inexact. In addition, glomerular filtration does not cease during stopped flow, and on release of stopped flow the proximal tubular fluid concentrations are necessarily further modified as the fluid passes through the distal nephron prior to collection.

Thus, the technique does not have the precision that micropuncture or perfusion of isolated tubular segments allows. Nonetheless, numerous relevant experiments have been conducted using this particular technique (Samiy *et al.* 1960; Murdaugh and Robinson 1960; Swanson and Hakim 1962).

1.3 The animal model

The animal model chosen for this experiment was the pig. There were two major reasons for its use. Firstly, the Landrace x White is an animal model which is in common use at the University of Cape Town Medical School. Expertise exists on procedures in that animal at the Department of Surgery's Animal Laboratory.

The second reason for using the pig as an animal model is because of the similarities between pig and human renal physiology. In particular, nephron type, maximum urine concentration, and maximum urine/plasma osmolal ratios have been shown to be similar in both (and both are quite different from the dog) (Nielsen *et al.* 1986).

Munsick *et al.* (1958) showed the pig to be a good model for renal studies. They also found that the clearance of endogenous creatinine was consistently lower in pigs than the clearance of inulin. The reason for this was unexplained. Subsequently Suarez *et al.* (1968) confirmed the similarities between the pig kidney and the human kidney but also noted that the manner in which the pig cleared creatinine is still unresolved.

1.4. Introduction to aluminium assay

1.4.1. General

The two major areas of concern with regard to Al assay are avoidance of contamination by the element, and the technique of using graphite furnace atomic absorption spectroscopy (GFAAS).

There is wide variability in the literature regarding "normal" values for human serum and urine Al. In a survey of data published from 1974 to 1984 the range of

serum Al values was between 2.1 µg/L and 42 µg/L; the range of urine Al values was between 4.7 µg/L and 1700 µg/L (Cornelis & Schutyser 1984). The issue arises as to whether this large range is due to a wide variability in the "normal" range or whether it is due to differences and problems in analytical technique including care taken to prevent contamination (Berlyne 1985; Adan 1985; Burnatowska-Hledin and Mayor 1985).

1.4.2. Prevention of aluminium contamination

Contamination of Al is a major factor in the accuracy of assaying the element at low concentrations (Cornelis & Schutyser 1984; Frech 1982; D'Haese 1985; Burnatowska -Hledin and Mayor 1985).

1.4.3 Dust control

A study conducted by Cornelis & Schutyser (1984) showed that a major source of Al contamination was from the air in the environment.

Figures quoted for Al contamination from air fall-out were 179 µg/m^2/d outdoors, and 8.2 µg/m^2/d in an analytical laboratory. There was a 9-fold decrease in the Al content of a clean-air laboratory as compared to the usual analytical environment (Cornelis & Schutyser 1984).

Frech *et al.* (1982) took blood samples from 43 normal individuals in a hospital, taking the usual precautions, and reported a mean blood Al level of 7.5 (SD ± 6.41) µg/L. In a separate study, using the same technique they took blood samples from 11 different volunteers in a dust-free atmosphere and found the mean blood Al to be 1.6 (SD ± 1.29) µg/L. Analyzing this discrepancy they concluded that contamination during sampling could have been a factor in the higher normal values found in the samples collected in the hospital.

1.4.4. Preparation of containers

The containers used may cause analytical problems either by increasing the Al content of the sample due to Al contamination of the vessel, or alternatively by decreasing the Al content by promoting adsorption of the element onto the wall of the container. These sources of analytical error are dependent both on the nature of the sample and the container (Cornelis & Schutyser 1984).

In order to decrease Al contamination of containers or other laboratory equipment these should be washed thoroughly, and stored for at least 12 h in a 10% nitric acid solution and then rinsed for several hours in Al-free water.

Moreover, the containers are composed of materials which may contain Al. Laboratory glassware may contain 100 µg Al/g, whereas high pressure polyethylene contains between 0.01 and 0.1 µg Al/g (Cornelis & Schutyser 1984). In general, plastics contain less Al than glass.

It is also possible for Al to adsorb to the walls of containers, particularly if stored for long periods of time. Refrigeration of the samples (rather than freezing) may prevent losses of Al in this way (Cornelis & Schutyser 1984).

1.4.5. Solutions

Water can be a major source of contamination in the cleaning of equipment, containers, and making up solutions. Water Al content varies in different areas (Ward *et al.* 1978). The only way to ensure that the water source is Al-free is to use double-deionised or reverse-osmosis water prepared by an appropriate filtration system. Only this Al-free water should be used in all aspects of Al measurement, including washing of containers and making up of solutions.

It has been well established that tap water and distilled water may contain appreciable amounts of Al (Ward *et al.* 1978). The use of double-deionised or reverse osmosis

water is necessary to produce water with Al levels of <10 µg/L.

Reagents used should be of a high degree of purity, with a minimum Al content.

1.4.6. **Analytical techniques**

Problems may be experienced in the measuring of urine or serum Al by GFAAS because of matrix effects ie the effects of substances other than Al in the sample being analysed. As a result samples are often "pre-treated" to facilitate their measurement. The simplest and most effective method appears to be by diluting the sample with water, although Triton-X and other techniques have been used (Frech *et al.* 1982; D'Haese *et al.* 1985). The preparation of a suitable standard is also important. It is important that the standards have the same constituents, other than Al, as the sample being studied: ie that sample-based standards be used.

A number of methods exist for measuring Al in various fluids and tissues (Burnatowska-Hledin and Mayor 1985; Berlyne 1985). These include neutron activation, X-ray microanalysis, inductively coupled plasma (ICP), atomic emission spectrophotometry, atomic absorption flame photometry and graphite furnace atomic absorption spectrometry (GFAAS).

GFAAS is the method of choice in many dialysis centres measuring Al in serum, urine and dialysis waters (D'Haese *et al.* 1985; Gorsky and Dietz 1978; Frech *et al.* 1982). Various instrument settings and heating programs exist, depending on local experience with the particular machine used, the sample pre-treatment performed and the sample to be analysed.

An Autosampler was found to be an essential part of the equipment for a number of reasons. Firstly, it allowed accurate pipetting from 5 µl to 50 µl, in 1 µl increments. Secondly, the pipetting is done accurately by the pipetting arm so that the sample pipetted is deposited at exactly the same spot in the graphite tube, which increases accuracy.

1.4.7. Choice of units

The concentration of a substance may be expressed as µg/L or as mmol/L. The S.I. system allows the expression of concentrations in both these formats (Reynolds 1989). Considering the low concentrations of Al usually discussed in this section µg/L has been used. The conversion factor for Al is

3.706×10^{-5} . µg/L = mmol/L.

2. MATERIALS AND METHODS

2.1. Aluminium assay

2.1.1. Prevention of aluminium contamination

As reviewed in the previous section, the main contaminants of Al arise from atmospheric dust, glass, metals and reagents.

Dust control

All containers and tubes once cleaned were kept stoppered to prevent dust contamination. Whenever possible the tubes were opened in a laminar flow hood. If circumstances did not permit the use of a laminar flow hood, the tubes were left open for the shortest possible time. Solutions were made up in the laminar flow hood. Once containers had been cleaned and required drying, this was done by air drying them in the laminar flow hood.

Preparation of containers

Only plastic containers and equipment were used. No glassware was used other than the volumetric flasks required to make up stock solutions. All containers and equipment used were cleaned (after washing) by immersing

overnight in 10% nitric acid (AnalaR, BDH, Ltd) and then rinsing for a few hours in Al-free water. The containers were then dried in a laminar flow hood. Periodically, containers were tested to ensure that they were Al free. The nitric acid and water used as cleaning solutions were kept in plastic buckets with tight fitting lids. These solutions were changed every few weeks.

Solutions

Tap water and distilled water may contain considerable amounts of Al. Only Al-free water was used throughout all the experimental stages of this research (eg washing of equipment, making up solutions, dilution of samples). Al-free water, repeatedly tested to contain non-detectable levels of Al, was obtained from two sources:

a) A Milli-Q Water filtration system
b) Commercially as "Sterile Water for Irrigation" (Cat No AFB 7114, Sabax Ltd, Johannesburg).

All solutions were made up using Al-free water in a laminar flow hood. The Maintalyte Solution (Cat No AFB 2774, Sabax Ltd, Johannesburg; containing electrolytes and 10% dextrose in water) and 0.9% saline solution (Cat No AFB 1324, Sabax Ltd, Johannesburg) were also assayed and found to have non detectable levels of Al.

High purity grade reagents were used and were checked periodically to ensure that they had not become contaminated with Al.

Miscellaneous

Surgical gloves were not used during the collection or analysis of the samples as the powder that is used to coat them contains Al. Samples were stored at 4°C until assayed.

Plastic, disposable, acid-washed pipette tips were used to measure the fluid aliquots. The plastic auto-sampler cups for use in the GFAAS were cleaned as described above, and stored in a closed plastic container until ready for use.

The Al standard used was "Aluminium nitrate Standard Solution for AAS" (SpectrosoL, Cat No 14031 4S, BDH Chemicals Ltd, Poole, UK.). This solution contained 1 mg Al/ml or 1 000 ppm or had a concentration of 37.1 mmol/L of Al. This solution was then appropriately diluted to serve as standards for the GFAAS determinations. The standard solutions were kept in plastic vials and made up fresh every 2-3 weeks.

2.1.2. Graphite furnace atomic absorption spectroscopy (GFAAS) technique

Equipment

Perkin-Elmer 5000 Atomic Absorption Spectrometer

Perkin-Elmer HGA 500 Programmer

Perkin-Elmer AS-40 AutoSampler

Perkin-Elmer 56 Recorder

Pyrolytically-coated graphite furnace tubes

Sample preparation

The sample volume in all the urine assays was 20 µl. Urine samples were diluted 1:2 with water. In cases where the level of Al was too high the dilution factor was increased appropriately.

Setting-up of GFAAS

The GFAAS technique was carried out according to methodology recommended by Prof M Orren, Associate Professor and Head of the Department of Analytical Chemistry at the University of Cape Town.

The samples were assayed in an argon gas flow. The injection port-hole was cleaned to remove any residue from previous samples. A new graphite tube (or a tube

that had previously only been used for Al estimations) was then inserted. If a new tube was used it was "conditioned" prior to use by firing it at gradually increasing temperatures until 2700°C was reached.

The settings on the spectrometer were: aluminium hollow cathode lamp wave length 309.3nm, slit 0.7nm, lamp 9mA. A furnace temperature of 2400°C was then selected on the Programmer. While depressing the "Manual Temperature" button the calibrating dial in front of the furnace was turned until the red and green lights were both on together. The locking lever was then used to fix the settings.

Once the spectrometer was set up as above, the lamp was adjusted to provide the optimum reading. The furnace alignment was also adjusted using the screws provided, so that the furnace was directly in the path of the light beam.

The standard operating conditions were as follows:

	Temp(°C)	Ramp(sec)	Hold(sec)	Gas Flow(ml/min)
DRYING	120	10	25	300
ASHING	1200	10	10	300
ATOMISING	2700	1	3	10
CLEANING	2700	1	3	300

Calibration of the GFAAS was done prior to each run. The calibration was performed on sample based standards, using a sample from the batch to be assayed and spiking the sample with known quantities of aluminium. The Autosampler was used in all instances.

VALIDATION OF ASSAY

CALIBRATION CURVE

The instrument produced a linear relationship between GFAAS units and Al concentration from 0-100 µg/L, the equation of the calibration curve being $y=0.00413x$ x (SE $\pm$ 0.00015) with a correlation coefficient of $r=0.9987$ (see Figure 2).

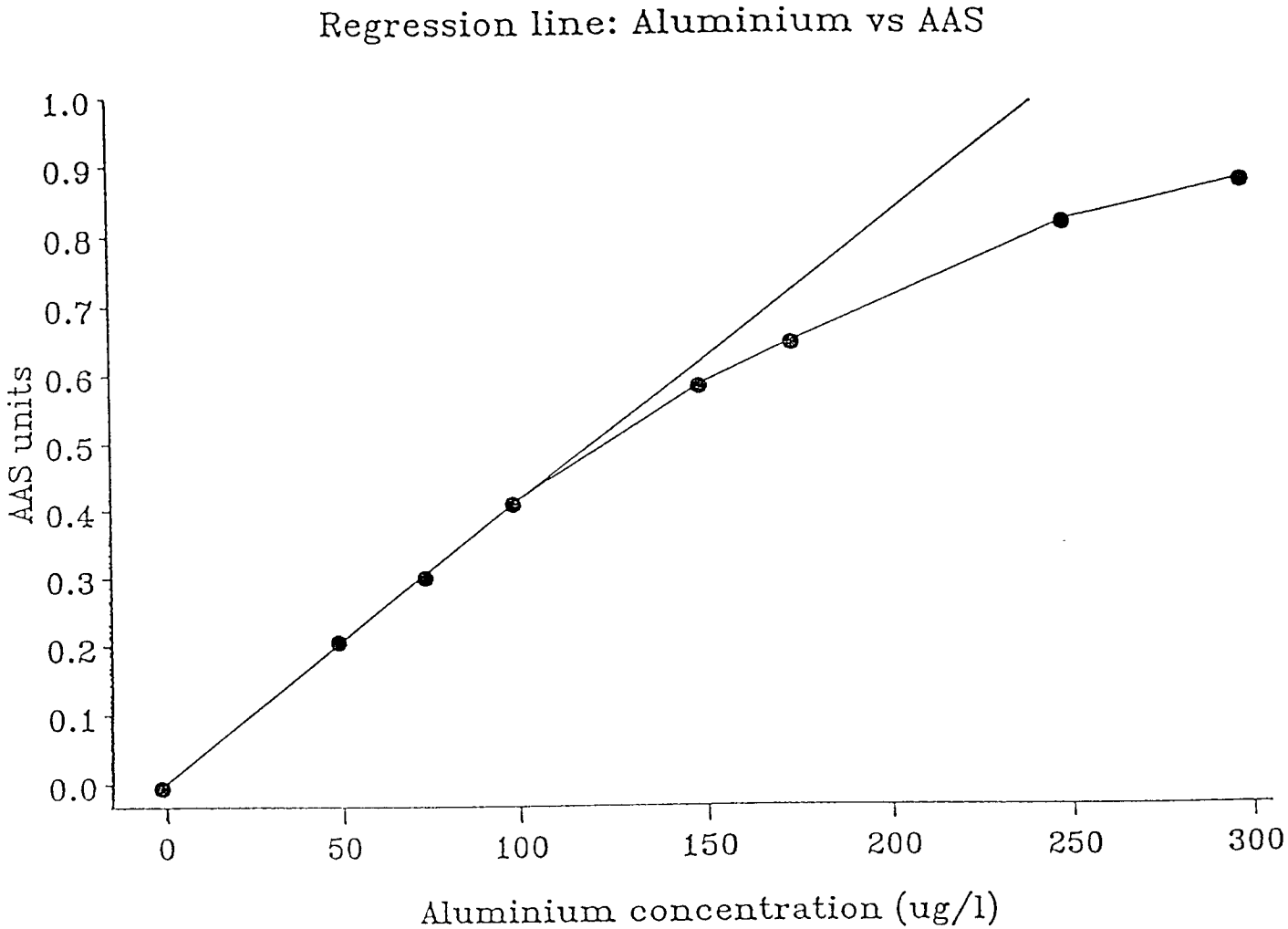

FIGURE 2: Regression line of aluminium concentration versus graphite furnace atomic absorption units.

Repeatability

Six blank samples spiked with 200 µg/L Al showed a coefficient of variation of 1.94%.

Sensitivity

For purposes of this study Al concentrations less than 10 µg/L were considered to be "non-detectable".

Effect of different concentrations of calcium and phosphate on the Al assay

To determine the magnitude of interference (if any) from Ca or Pi, known quantities of Al were spiked with increasing concentrations of Ca or Pi.

Calcium

A 50 µg/L Al standard solution, spiked with calcium chloride, yielded:

Calcium (Calcette assay) mg/dL	Al (GFAAS assay) µg/L
0.00	50
0.08	50
0.30	48
0.79	48
1.17	48
1.65	50
2.00	55
2.46	53
3.19	55
4.01	56

For details of Calcette assay please see Section 2.2.5.

Phosphate

A 100 µg/L Al standard solution, spiked with phosphate, yielded:

Phosphate	Al (GFAAS assay)
mg/dL	µg/L
0.00	100
0.1	100
1.0	109
2.5	109
5.0	108

2.2. Other assays

2.2.1. Choice of units

According to the S.I. system the concentration of a substance may be expressed as mg/dL or mmol/L (Reynolds 1989). The conversion factor for calcium is y mg/dL = y/4 mmol/L. The conversion factor for phosphate is y mg/dL = y/3.096 mmol/L.

2.2.2. Inorganic phosphate (Pi) assay

The assay used was based on the method described by Chen *et al.* (Chen *et al.* 1956).

Equipment and materials

A Beckman Model 35 spectrophotometer (Beckman Instruments Inc., Irvin, California) was used.

REAGENT C was made up as follows:

1 vol 6N sulphuric acid
2 vol distilled water
1 vol 2.5% ammonium heptamolybdate
1 vol 10% ascorbate

Standards

Urine aqueous standards were used containing 3.33 mg Pi/dL and 10 mg Pi/dL and used as appropriate.

Serum standards containing 3.6 mg Pi/dL were obtained commercially (Versatol, General Diagnostics, Morris Plains, New Jersey, USA).

Method

Initial urine Pi preparation

Twenty microlitres urine (on occasion with low absorbances 40 µl urine was used) and 100 µl of 10% trichloroacetic acid were mixed with 1 ml Reagent C in a test-tube. The resultant solution was processed as described below. Standard phosphate solutions of 3.33 mg Pi/dL and 10 mg Pi/dL were prepared.

Initial serum Pi preparation

Forty microlitres serum (20 µl serum was found to give absorbances that were too low) and 200 µl of 10% trichloroacetic acid were mixed together in a test-tube. After standing for 10 minutes the solution was centrifuged and 100 µl of the supernatant was added to 1 ml Reagent C.

The resulting solution was processed as described below.

Final processing of urine and serum samples

After the urine and serum samples had been prepared as described above they were treated similarly. The samples were incubated in a water-bath at 37°C for 90 minutes. The samples were then placed in the spectrophotometer and

read at an absorbance of 700 nm. Each specimen was assayed in duplicate. By using the appropriate standards the absolute values of Pi were obtained.

2.2.3. Creatinine assay

Equipment

Glucose, Urea, Creatinine Analyzer Model 919 using Auto-sampler Model 461 (Instrumentation Laboratory, Inc, Lexington, Massachusetts, USA).

Method

The auto-analyzer was used following the manufacturers' instructions. The machine required 240 µl per assay. Where necessary the sample was diluted with water.

2.2.4. Inulin assay

The inulin assay was based on the method of Young and Raisz (1952).

Equipment and materials

A Beckman Model 35 spectrophotometer (Beckman Instruments Inc., Irvin, California) was used.

Anthrone Reagent

Two grams of anthrone was placed in a 500 ml volumetric flask and dissolved in 125 ml concentrated sulphuric acid. The volume was then adjusted to the mark with diluted sulphuric acid (2:1). This procedure was carried out in a water bath.

Method

Initial urine preparation

The samples were prepared in duplicate.

Ten µl of urine was mixed with 200 µl of water and 10 µl of 4N sodium hydroxide in a test-tube, and the solution was heated for 20 minutes at 95°C in a heating block. The tubes were inverted to collect the condensed fluid.

An amount of 50 µl was removed and placed in a clean glass tube.

Standards of 12.5, 50, 100 and 200 mg/dL inulin were processed in the same way.

Initial measurement in plasma

The samples were prepared in duplicate.

An amount of 100 µl of serum was diluted with 0,980ml water in a test-tube. To this was added 75 µl of 10% zinc sulphate and 75 µl of 0,5N sodium hydroxide.

Standards of 25, 50, 100, 200 and 400 mg/dL inulin were processed in the same way.

After centrifugation 400 µl of supernatant was removed and 100 µl 4N sodium hydroxide was added. At the end of this period, 200 µl was removed and placed in a clean glass tube.

Final processing of urine, serum samples and standards

After the initial preparation of the urine and serum samples, the tubes were placed in an ice-water mixture, and 1 ml of the cold anthrone reagent was added. The tubes were agitated while in the ice-water to ensure thorough mixing. They were then placed in a 75°C water-bath for 5 min after which they were cooled by re-immersion in ice water. The samples were read in the spectrophotometer at 630 nm after waiting for at least 15 minutes.

2.2.5. Calcium assay

Equipment

Calcette - Automatic Calcium Analyzer (Model No. 4008, Precision Systems Inc, Sudbury, Massachusetts). The Calcette measures total calcium by a fluorescence technique. Reagents and standards as supplied by the manufacturers.

Method

The "Calcette" Automatic Calcium Analyzer was used in accordance with instructions from the manufacturers. The procedure was the same for urine or serum. The machine required 80 µl per assay and the assays were all done in duplicate. The standard used was 10 mg/dL aqueous calcium as supplied by the manufacturer. Once the machine was calibrated, readings were obtained directly from the digital read-out.

2.2.6. Sodium assay

Equipment

Sodium Flame Photometer Model 543 (Instrumentation Laboratory Inc., Lexington, Massachusetts, USA).

Method

Samples were read directly from the Flame Photometer, after previous calibration.

2.3. The experiment

2.3.1. Setting up the animal model

Landrace x Large White pigs were obtained from the University of Cape Town Animal House. Both male and female pigs were used, with a median weight of 24.5 kg (range:19 to 40 kg). They were maintained on a balanced diet, were in good health and had not been used in any experiment previously. Fresh pigs were used for each experiment conducted.

They were fasted overnight prior to the day of the experiment to avoid anaesthetic complications. On that morning the experimental pig was transferred to the UCT Department of Surgery's Animal Theatre where the procedures took place.

Once in the animal theatre the pigs were weighed and then anaesthetised using thiopentone sodium induction and a nitrous oxide/oxygen mixture (at a flow rate of 4 l/min and 2 l/min, respectively). Respiration was maintained

via an endotracheal tube using a volume cycled respirator.

Once the pig was anaesthetised a plastic catheter was inserted into an external jugular vein for infusion and subsequent injections. Another plastic catheter was inserted into the external carotid artery, for collection of blood samples, and monitoring of blood pressure to note undue fluctuations. Temperature was not monitored.

A mid-line laparotomy was then performed. A plastic catheter was inserted into the right ureter, with its tip sited in the renal pelvis. The catheter was then tied firmly in place; its free end passed through the laparotomy incision into a measuring cylinder.

Urine from the left kidney collected in the bladder during the experiment, and in order to prevent bladder distension, pain and subsequent neurogenic stimuli, the bladder was drained by means of another catheter. The urine drained from the bladder was discarded and did not play a part in any of the results shown. The laparotomy wound was partly closed, with the abdominal contents covered with damp swabs in order to minimise fluid loss from evaporation.

Once all the appropriate catheters had been inserted a solution containing 10% dextrose water was infused

intravenously at a rate sufficient to produce a diuresis. In Pigs I to VII the solution used was a commercially available liquid Maintalyte (Sabax, Ltd). In Pig VIII 0.9% saline (Sabax, Ltd) to which dextrose had been added to achieve a final dextrose concentration of 10%, was used.

2.3.2. The typical experiment

Once the animal model had been set up as described above and there was a copious diuresis ensuing from the ureteric catheter the experiment was ready to begin.

Experimental plan

A total of eight pigs was used in these experiments.

Pigs I and II were used to demonstrate endogenous secretion of Al, and also acted as controls.

Pigs III and IV were used to determine a suitable dose range of Al to be used in subsequent experiments.

Pigs V,VI,VII and VIII received 0.1 mg Al/kg b.w.. In these four pigs seven regular and four interrupted stop-flows were performed. The inulin clearances were determined in pig VIII.

Urine free-flow periods

The urine free-flow periods are those during which urine flowed freely before and after the stop-flow experiments. During these periods blood and urine samples were collected. From these samples plasma and urine creatinine and occasionally inulin were assayed. These assays were used to determine the single kidney creatinine clearance.

Stop-flow experiments

Regular stop-flow

After a variable number of free-flow measurements had been taken a stop-flow experiment was then performed. The ureteric catheter was clamped for five minutes and when the clamp was released the ensuing gush of urine was collected serially in 40 tared, labelled 1.5 ml Microfuge tubes. Each tube received about 0.5 to 1.0 ml of urine, and was promptly sealed to minimise Al contamination.

The volume of urine in each tube was determined by the subsequent weighing of the tubes and equating a gram with a millilitre of urine.

Since it was found that each tube did not contain enough urine to enable all the assays to be done, the tubes were consecutively paired and the data presented as such.

Thus, every alternate tube was used for Al measurement; the remaining tubes were used for the sodium, phosphate, calcium and creatinine assays. The results are displayed as 20 data points, each representing the concentration in the tubes of the various substances at that point.

Interrupted stop-flow

Four interrupted stop-flows were conducted in Pigs V and VIII. In each case, the catheter was occluded for five minutes, the clamp then released allowing approximately 10 ml of urine to escape, and the ureteric catheter then occluded for a further five minutes.

After this second occlusion, stop-flow samples were collected serially as described in the regular stop-flow section.

Aluminium injection

During the course of the experiment in each pig a test substance was injected as a slow IV bolus. Only two solutions made up in sterile water, were injected aluminium chloride and hydrochloric acid. Hydrochloric acid was chosen as the control substance, as it could suitably be matched to the pH of the Al and it also contained a chloride ion.

Pigs I and II received 0.037 ml/ kg b.w. of the hydrochloric acid solution.

Pigs III and IV received 1.0 mg/kg b.w. and 0.5 mg/kg b.w. respectively of the aluminium chloride solution.

Pigs V,VI,VII and VIII received 0.1 mg/kg b.w. of the aluminium chloride solution.

2.3.3. **Creatinine and inulin clearance studies**

These clearance studies were conducted in the usual fashion. A timed collection of urine was obtained, and by measuring the serum and urine creatinine (or inulin) the clearance was obtained. Important points with regard to the experiment were:

1. The timed period was 5 minutes (measured with a digital stopwatch).

2. The urine was collected from the ureteric catheter into a graduated measuring cylinder. After the volume was measured a sample was taken for creatinine assay.

3. The blood sample was obtained from the arterial line via a three-way tap. The sample was then centrifuged and the serum stored.

4. The clearance values obtained apply to only one kidney, as the urine was collected from the single ureteric catheter.

Using this information clearances were calculated as:

$$\text{CREATININE OR INULIN CLEARANCE} = \frac{U.V}{P} \text{ ml/min}$$

where

U = urine conc of creatinine (μmol/L) or inulin (mg/dL)
P = serum conc of creatinine (μmol/L) or inulin (mg/dL)
V = urine volume passed in one minute (ml/min).

3. RESULTS

3.1. General

Details of the experimental plan in each of the eight pigs are set out in the Appendix, including the timed sequence of events and the weight of each pig. The free-flow studies are designated numerically, and the stop-flows alphabetically. For greater clarity an (R) or (I) after the stop-flow denotes a regular or interrupted stop-flow respectively. For example, S-F.V.A(R)

designates a stop-flow in pig V, which was the first one conducted in that pig (ie A), and which was a regular stop-flow (ie R).

The median weight of the pigs was 24.5 kg (range: 19-40 kg).

In the control pigs I and II, there were 18 free-flow studies and 4 stop-flows (all "regular"). In pigs III and IV, the pigs used to establish the dose range of Al, there were 17 free-flow studies and 6 stop-flows (all "regular"). In pigs V, VI, VII and VIII there were 34 free-flow studies and 13 stop-flows (9 "regular" and 4 "interrupted"). These last 13 stop-flows formed the basis of the final analysis.

3.2. Urine free-flow studies

3.2.1. Single kidney creatinine and inulin clearances

In all eight pigs the single kidney creatinine clearance was found to be 25.2 ml/min (range: 16.3-34.7 ml/min), and in pig VIII where inulin clearances were also done, the inulin clearance was 34.0 ml/min (range: 29.5-37.6 ml/min). The median ratio of creatinine to inulin clearance was 0.69 (range: 0.58-0.78). The median urine flow rate from a single kidney during the clearance collections was 4.7 ml/min (range: 3.0-9.0 ml/min).

The sodium and phosphate maximal reabsorption sites, are arbitrarily designated 0% and 100% of the intratubular volume respectively, and act as markers for tubule length. The median tubular urine volume (ie between sites 0% and 100%) collected in the series of stop-flows was 11.4 ml (range: 9.2-16.2 ml).

In the eight pigs, the creatinine clearances were determined on 3 (range: 2-6) occasions prior to injection with Al or control, and on 5 (range: 4-8) occasions afterwards.

3.3. Stop-flow studies

3.3.1. Control experiments

Measurable quantities of aluminium were not detected in the urine, either before or during stop-flow.

3.3.2. Dosage experiments

The initial quantities (1 and 0.5 mg Al/kg b.w.) of aluminium used to determine the final dose to be administered were unnecessarily large in terms of what could be measured. Consequently, 0.1 mg Al/kg b.w. was arbitrarily chosen as the standard dose in the remaining animals.

3.3.3. Aluminium experiments

The results from the stop-flow experiments in four pigs dosed with 0.1 mg Al/kg b.w. are presented.

The fractional excretion of filtered creatinine along the length of a "tubule" was calculated in those four stop-flows in which inulin was measured concurrently. Peaks or troughs in creatinine along the length of the "tubule" were not detected.

The pooled results for the aluminium excretion in the stop-flows are displayed in Figure 3 (a). In each stop-flow experiment, the Al/creatinine ratio in each intratubular sample was expressed as a percentage of the peak Al/creatinine value found. The individual stop-flows demonstrated patterns of excretion and reabsorption similar to those illustrated in Figure 4a) and b) of S-F.V.A(R). The sodium, phosphate, calcium and aluminium concentrations are represented as relative to that of creatinine concentration. Figure 4 (a) demonstrates the sites of maximal reabsorption of sodium phosphate and calcium. Figure 4 (b) demonstrates the creatinine and the aluminium/creatinine concentration along the tubule. In the series of experiments the median calcium/creatinine minimum was at site 14% (range: 11 to 27%) and the median aluminium peak was at site 12% (range: -27 to +31%).

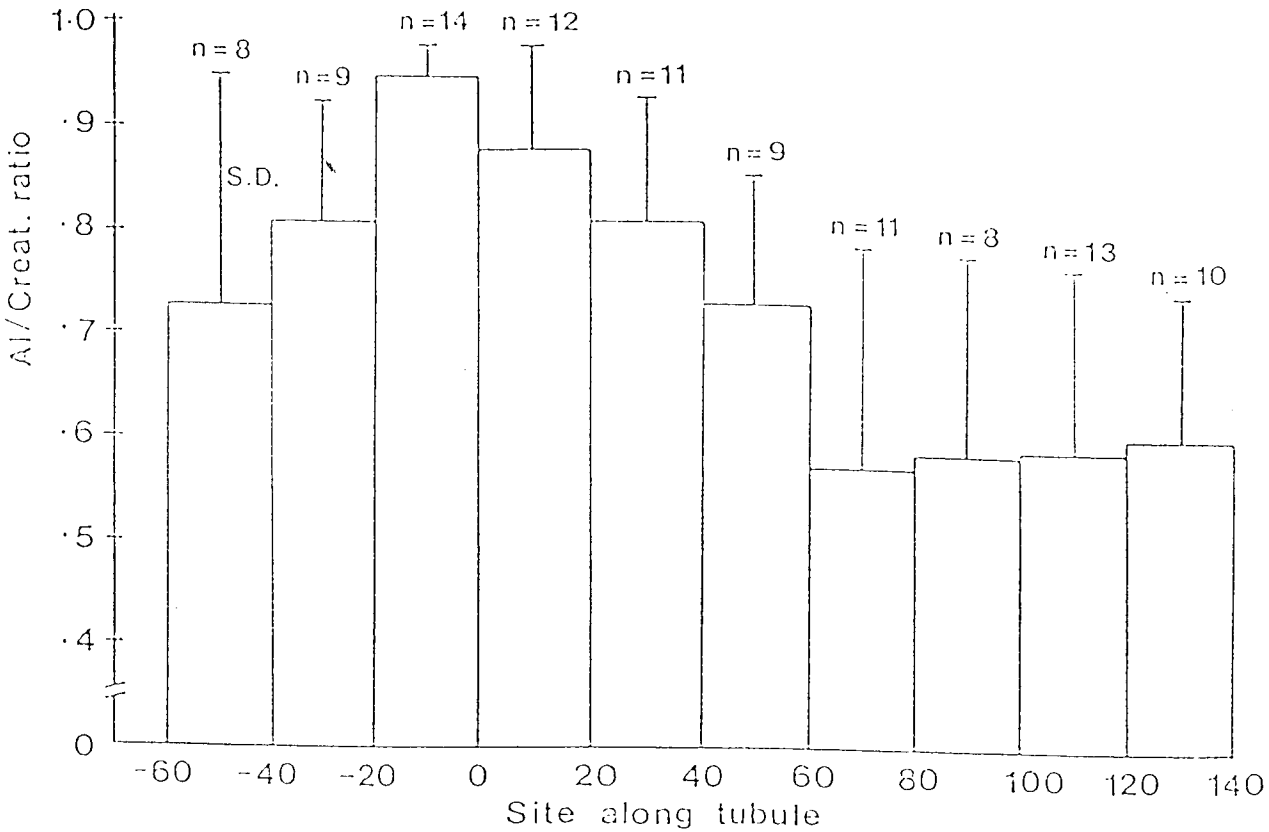

FIGURE 3a: Data from seven stop-flow experiments in 4 pigs. In each stop-flow experiment, the Al/creatinine ratio in each intratubular sample was expressed as a ratio of the peak Al/creatinine value found. The figure depicts the mean (and SD) ratios found along the length.

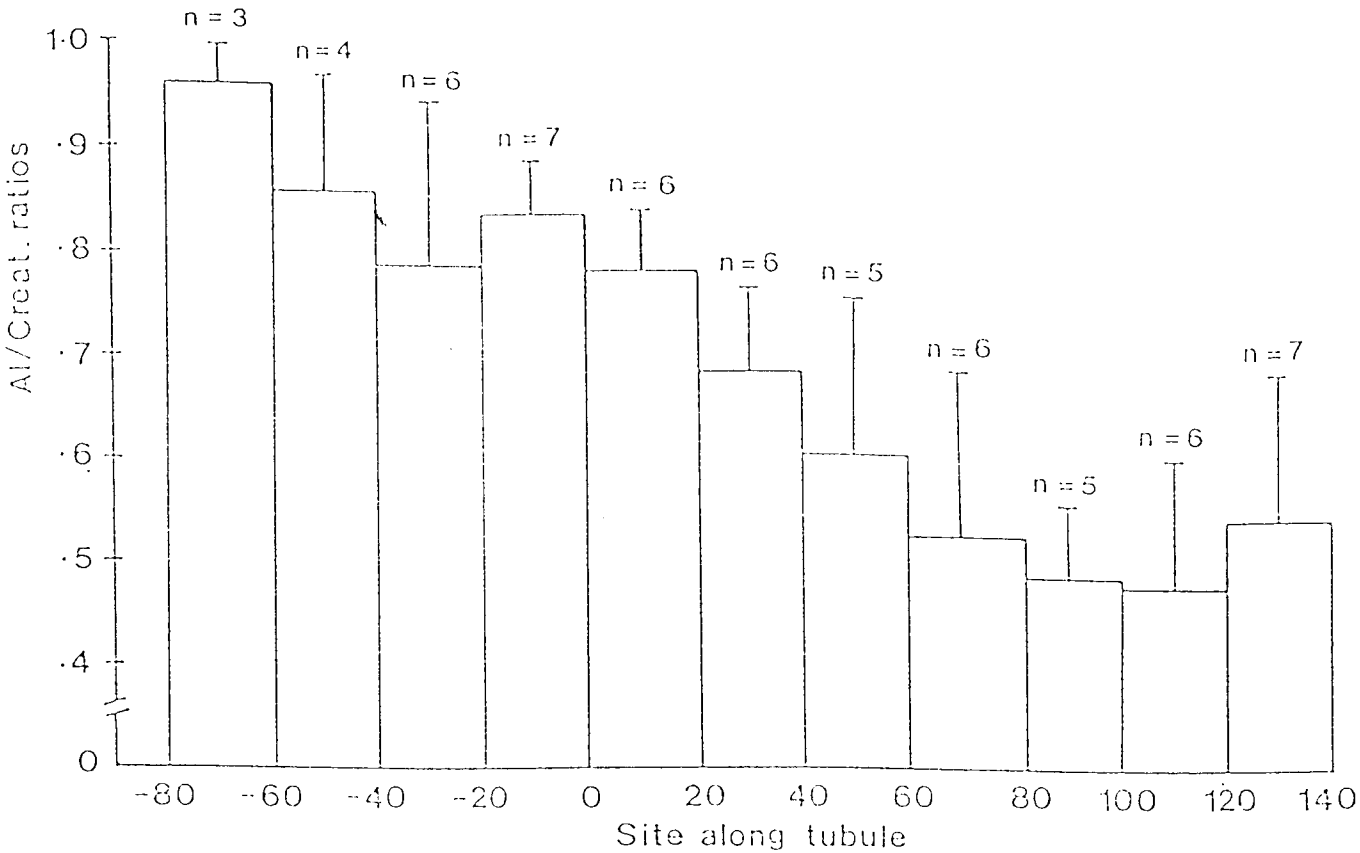

FIGURE 3b: Data from four interrupted stop–flow experiments in 2 pigs. In each interrupted stop–flow experiment, the Al/creatinine ratio in each intratubular sample was expressed as a ratio of the peak Al/creatinine value found. The figure depicts the mean (and SD) ratios found along the length.

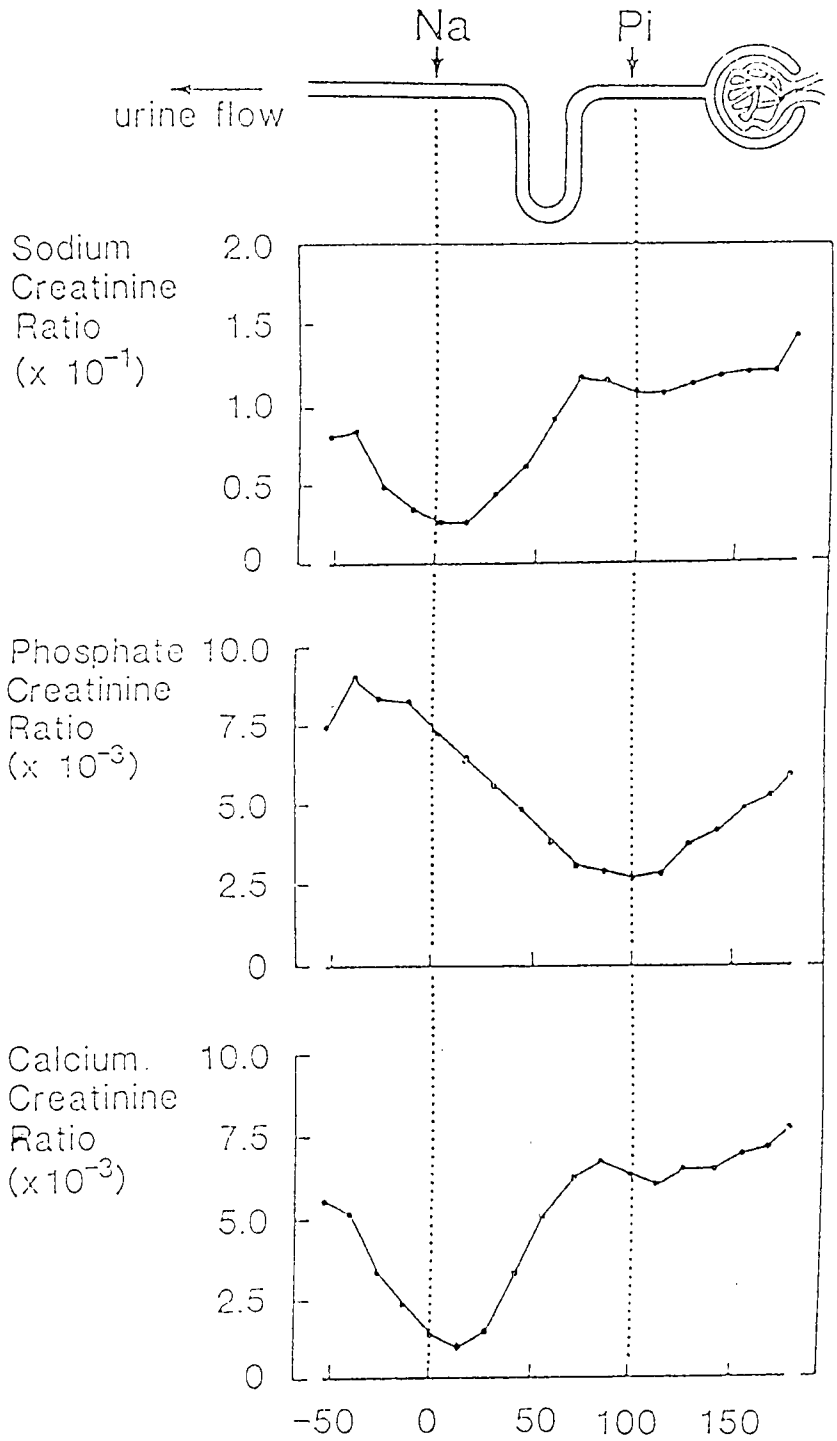

FIGURE 4a: Graphs demonstrating the ratios of sodium/creatinine, phosphate/creatinine, and calcium/creatinine during stop-flow. The free-flow urine values immediately prior to this experiment for sodium/creatinine, phosphate/creatinine and calcium/creatinine were 0.12, 0.01, and 0.0069, respectively.

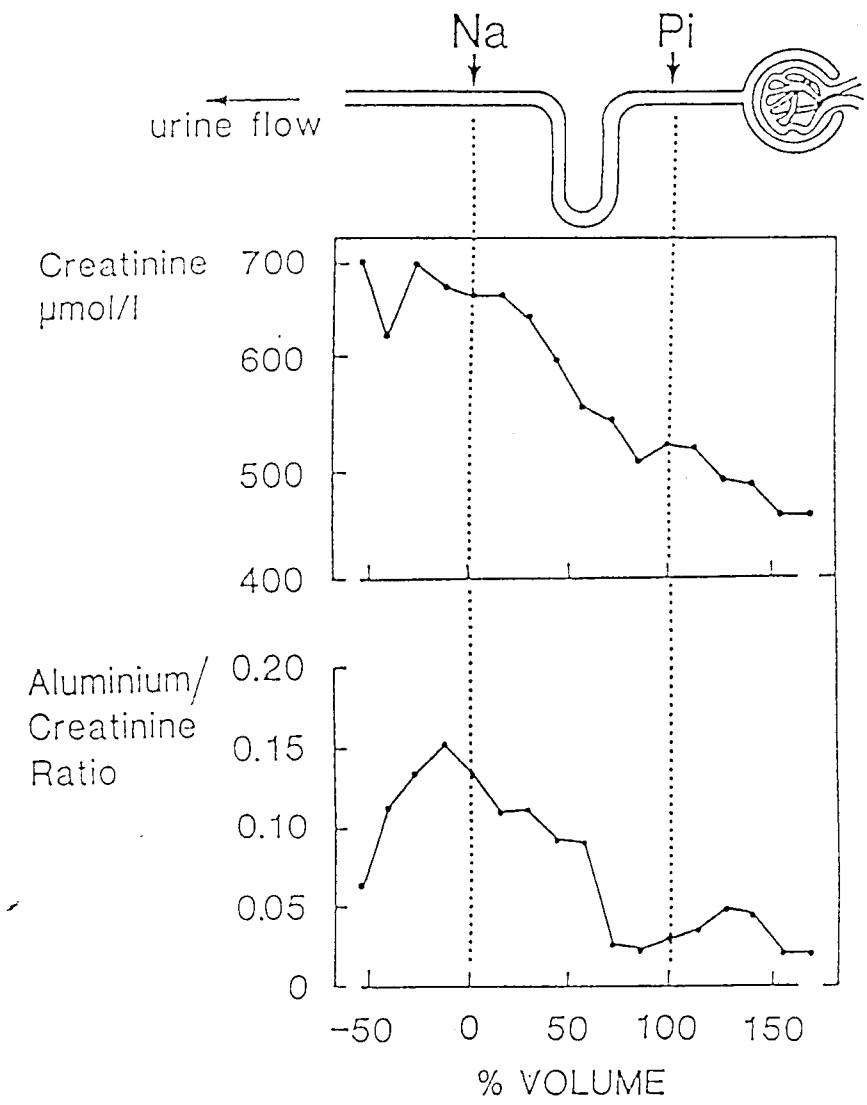

FIGURE 4b: Graphs demonstrating the creatinine and the Al/creatinine ratios during stop-flow. Free-flow urine values immediately prior to this experiment for Al/creatinine and creatinine were 0.042 and 564, respectively.

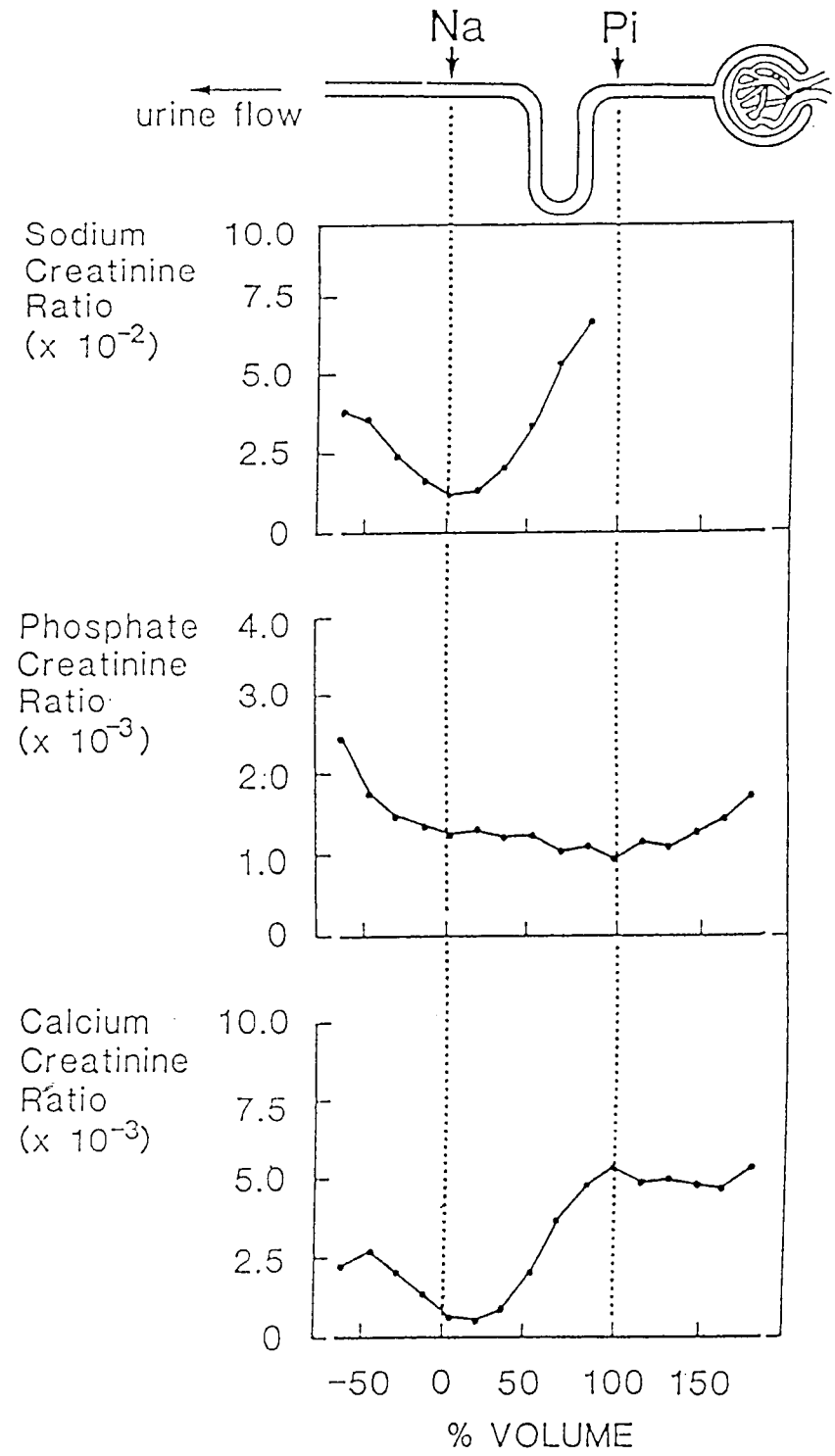

FIGURE 5a: Graphs demonstrating the ratios of Na/creatinine, phosphate/creatinine and calcium/creatinine during interrupted stop-flow.

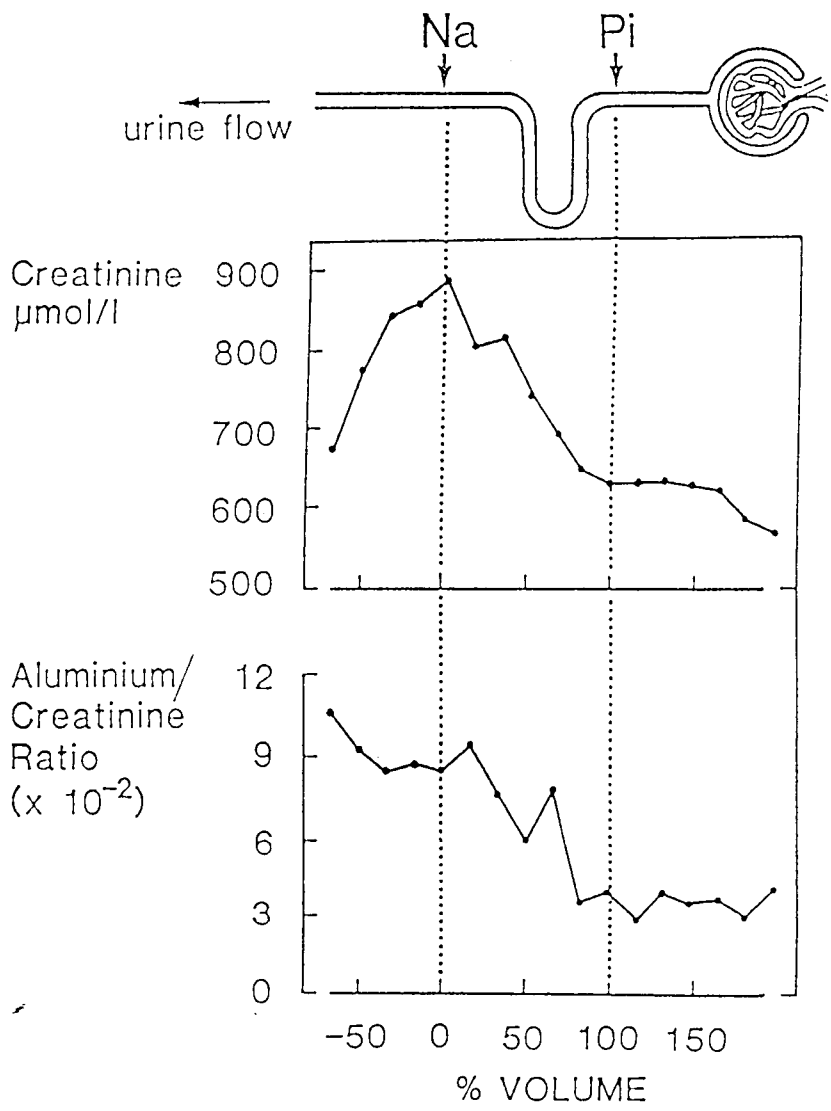

FIGURE 5b: Graphs demonstrating the creatinine and the Al/creatinine ratios during interrupted stop-flow.

Four interrupted stop-flow experiments were conducted in two pigs. The pooled results (pooled in an analogous manner to Figure 3 (a)) for the aluminium excretion in the interrupted stop-flows are displayed in Figure 3 (b). The individual interrupted stop-flows demonstrated patterns of excretion and reabsorption similar to those illustrated in Figure 5 a) and b) of S-F.V.B(I). Interrupted stop-flow was done immediately after the regular stop-flow in Figure 4, on the same animal. The graphs in Figure 5 a) and b) demonstrate analogous features to those in Figure 4 a) and b), as set out in the above paragraph.

The results of the interrupted stop-flow demonstrate that aluminium excretion continues in the second half of the experiment. This excretion causes a peak of aluminium to become evident in the previous proximal low that has been shifted to lie opposite the aluminium excretory site in the distal nephron. Moreover, distal to the aluminium/creatinine peak shown in the stop-flow no further aluminium reabsorption or excretion occurs.

4. DISCUSSION

4.1 Discussion of the Literature

The kinetics of Al in humans and animals have been described. The kidney is known to be the major route of

excretion of the element. Despite this the way in which Al is handled by the kidney is still not clearly defined. It is known that the element is highly protein bound in plasma and that only about 3-5% is cleared in the urine. Previous to this current work it was not definitively known if the element was purely filtered by the glomerulus, or if reabsorption or secretion by the tubule takes place.

Only one paper was found where an attempt was made to elucidate mechanisms of tubular handling of Al (Burnatowska-Hledin *et al.*1985). This work has already been described in Introductory Section 1.1. They inferred that Al was reabsorbed in the proximal tubule because the excretion of Al increased in response to volume expansion but not to furosemide. The authors noted that a direct determination of Al by their technique was not possible.

Using the above as background the concept of using the stop-flow technique of Malvin *et al.* (1958a) was considered. This technique has in most instances been superseded by micropuncture and isolated perfused tubule methodologies which are more precise. A critique of stop-flow methodology is found in the Introductory Section 1.2.4. Nonetheless, this technique has been used extensively and produced results which were found to be comparable to data produced by other methodologies. Its

advantage in this study is the fact that it produces sample volumes that are adequate for GFAAS.

The interrupted stop-flow method was devised by Murdaugh and Robinson (1960) and is also described in the Introductory Section 1.2.3. The particular value of this technique is that it allows detection of possible secretion into the distal tubule of solutes with a low solute/creatinine ratio proximally. It increases the "signal/noise" ratio and so facilitates detection of possible ion exchange phenomena (eg sodium/hydrogen) which might otherwise be obscured by high concentrations. So for Al in particular, the distal Al/creatinine ratios can be seen to be still high after interrupted stop-flow despite the fact that the distal Al/creatinine ratio immediately after the release of the fluid after the preliminary stop-flow must have been low.

The experimental animal chosen for this series of experiments was the pig. The reasoning for this decision has already been noted (Section 1.3.) The similarity between the human and the pig kidney were important considerations in this decision.

Hohr *et al.* (1989) demonstrated in their isolated perfused rat kidney experiments that acutely administered Al did not affect general kidney functions, such as GFR, urine volume, the excretion of potassium and the fraction

reabsorption of sodium and glucose. This may be of significance in the stop-flow study suggesting that the acute administration of Al to the pigs (although a different species and dose range) would not interfere with their renal function.

The developing of the GFAAS technique was a major part of this research. To start with the samples had to be kept free of significant Al contamination. In the Al range of <10 µg/L contamination is more difficult to control but this experiment only considered values above this. Nonetheless, scrupulous attention was given to potential sources of contamination, as described (Section 1.4) in order to increase the accuracy. This involved care in the solutions used, the containers and avoiding environmental dust.

The GFAAS technique would appear to be the "gold standard" for measuring Al in the µg/L range. Various modifications of this technique have been described, but the one that was used was developed by Professor M. Orren of Analytical Chemistry, University of Cape Town (Personal communication). Sample based standards were used in order to minimize matrix (ie substances present in the sample other than Al) effects. The effects of phosphate and calcium on the assay were examined (Section 2.1.2) and found not to be critical.

Various other assays were performed for phosphate, creatinine, inulin, calcium and sodium (Section 2.2). These methodologies are well described in the literature and were routine clinical laboratory assays in many cases.

4.2 Discussion of experimental work

4.2.1. Urine free-flow periods

In order to establish the validity of creatinine as a marker of water reabsorption, inulin levels were determined concurrently in four stop-flows (2 regular and 2 interrupted). The GFR calculated by the creatinine clearance was consistently lower than when calculated by the inulin clearance, as had been noted in previous work in pigs (Munsick *et al.* 1958). The reason for this difference is not established. Examining the fractional excretion of filtered creatinine at various points along the nephron, no peak or trough pattern for that substance could be found. Moreover, division of a solute concentration by that of creatinine did not alter the site of the trough for that solute (e.g. sodium or phosphate), as previously classically described (Malvin *et al.* 1958a). The inulin and creatinine graphs were similar in the stop-flow experiments, and the Al/creatinine and the Al/inulin curves demonstrated the same finding of an Al peak in the distal nephron. As

such, it was felt that creatinine could be used as a marker for water reabsorption (although not for GFR).

4.2.2. Stop-flow

The concentration of many substances appears to rise along the tubule due to water reabsorption. To compensate for this water reabsorption, the concentrations of these substances were expressed as ratios relative to the creatinine concentration. A more exact index of excretion is the calculation of the fractional excretion of the filtered substance. However, for this calculation to be correct, the substance must be freely available for filtration through the glomerulus. This is not the case with Al which is extensively protein bound with only a small percentage existing in the free form in the plasma, thus making precise calculation of the fractional excretion impossible (Henry *et al.* 1984).

In all the stop-flow experiments the relationship of Na, phosphate, and calcium maximal reabsorption sites (against their concentration gradient) was as described by previous workers, confirming the general validity of the technique (Malvin *et al.* 1958a).

The median Al/creatinine peak occurred at site 12% (range:-27 to +31%) of intratubular length, near the site of maximal calcium and sodium reabsorption. The pooled

data for all the stop-flows are displayed in Figure 3a. This (together with the interrupted stop-flow data) indicates Al excretion in the distal nephron. The wide range in the Al/creatinine peaks may be due to the relative inaccuracy of the stop-flow technique. Moreover, since there are only about seven data points between sites 0 and 100%, the location of the site is relatively inaccurate.

Significant reabsorption of Al against its concentration gradient at the proximal nephron is not likely as the Al/creatinine levels at that site are in the same range as those in the free-flow tubular fluid (points > 150%).

Al excretion rather than secretion is noted, as an active mechanism for this process has not been demonstrated. The excretion of Al at the distal tubule could possibly be due to passive diffusion in a manner analogous to that postulated for Na in the collecting tubule (Stokes 1982).

4.2.3. **Interrupted stop-flow**

A typical interrupted stop-flow is demonstrated in Figure 5. Pooled data for the interrupted stop-flows are displayed in Figure 3b. Values were generally higher than in the regular stop-flow because of the greater duration of the procedure.

The difference between the Al/creatinine graphs in the stop-flow and the interrupted stop-flow can be explained as follows. During the first 5 min of the interrupted stop-flow an Al peak similar to that demonstrated in Figure 4 is likely to develop. By allowing some fraction of the intratubular fluid volume to escape, the entire column of tubular fluid is shifted toward the distal end of the nephron, so that the Al peak that was originally near the sodium reabsorption site is now moved downstream, while tubular fluid of low Al concentration is now opposite the site of the previous Al maximum concentration. The column of tubular fluid then remains stationary for a further 5 min in that position allowing further excretory and reabsorptive processes to take place. The pattern depicted in Figure 5 shows the composite effect of the second pattern superimposed on the first at the end of the experiment. It can be seen that further Al excretion has occurred near the sodium reabsorption site during the second half of the stop-flow, confirming the data of the regular stop-flows. Thus, the plateau of Al excretion from point -50 to 0% is formed by the Al/creatinine ratio present distally in the second half of the experiment replicating that in the first half, even though there was a low Al/creatinine ratio present initially.

SECTION C

TOXIC EFFECTS OF ALUMINIUM ON THE DNA OF KIDNEY CELLS IN CULTURE

SYNOPSIS

It is known that renal failure may lead to systemic aluminium (Al) accumulation and toxicity. The effect of Al on the kidney itself is not well established. The purpose of this section was to investigate the toxic effects of Al in vitro on the DNA of pig kidney cell line LLC-PK1, in an attempt to elucidate some of the mechanisms of action.

DNA synthesis was measured using 3H-TdR incorporation. Over increases of both time (9-72 h) and Al concentration (0.01-8.0 mM), 3H-TdR incorporation was diminished. Effects were evident at concentrations as low as 0.05 mM Al.

The production of DNA strand breaks was assessed by the increase in size of cell nucleoids (ie DNA in supercoiled form). Nucleoid size was analyzed in a Coulter Electronics Epics 753 Fluorescence Activated Cell Sorter interfaced with an MDADSII data acquisition and analysis system. After 90 min incubation with Al (over the concentration range 0.001-32 mM), an increase in nucleoid size was noted at concentrations above 0.05 mM.

The data demonstrate that Al exerts an effect on kidney cells in vitro which is expressed as diminished DNA synthesis and production of DNA strand breaks. These effects on DNA may have important long-term implications on various disease states associated with Al toxicity.

1. INTRODUCTION

1.1. Effects of aluminium on the kidney

A number of disease conditions, including dialysis dementia, renal osteodystrophy and anaemia are associated with Al accumulation, particularly in the setting of renal failure (Monteagudo *et al.* 1989). While renal impairment is a major determinant in the retention and accumulation of the element (Monteagudo *et al.* 1988), the effect of Al on the kidney itself has not been extensively documented.

Anatomically, using ion microscopy and X-ray microanalysis, Al has been shown to be concentrated inside the kidney proximal tubular cell lysosomes (de Galle 1981). Functional rat kidney lysosome damage caused by Al has also been demonstrated (Stein *et al.* 1987), and supports the localisation of the element in the kidney. Tissue analysis of dogs chronically injected with Al for 3-5 weeks demonstrated the kidney (together with the liver and spleen) as accumulating the greatest amount of the element (Henry *et al.* 1984).

At a functional level, rats injected intraperitoneally with Al had loss of renal concentrating ability compared with controls (Braunlich *et al.* 1986). This functional impairment was confirmed by Henry *et al.* (1984), who showed increases in serum creatinine, and decreases in exogenous creatinine clearance, after chronic injection of dogs with Al.

1.2. LLC-PK1 cell line

The purpose of this study was specifically to examine Al effects on DNA. The use of an in vitro system was considered appropriate for this purpose. The use of cell cultures, in general, allows the exclusion of many extraneous factors from the research in question. It is also useful in toxicological studies when the toxin is known not to be transformed by metabolic processes. It also reduces the need to engage in animal experimentation.

The choice of this particular LLC-PK1 cell line was made for a number of reasons (Hull *et al.* 1976). Firstly, these cells are of pig kidney origin and thus are pertinent to the work of the previous section where Al excretion by the pig kidney was demonstrated. Secondly, these cells represent a homogenous cell population with features of proximal tubular cells, and are well characterised (Cantiello *et al.* 1986; Holohan *et al.* 1988). Finally, the ability of these cells to grow in serum free conditions has been previously established (Hull *et al.* 1976) and this was of importance during the actual experiments in order to minimise Al binding to serum.

1.3. Effects of aluminium on DNA

It has been known for some time that a relationship exists between Al and DNA.

1.3.1. **Localisation of aluminium on DNA**

Berlyne *et al.* (1972) noted that rats severely intoxicated with Al had a marked reduction in the number of mitoses of the stratum basale of the corneal epithelium suggestive of decreased cell division and possibly a DNA effect. Intrathecal administration of Al to cats and rabbits showed Al to be histochemically demonstrable on nuclear chromatin (De Boni *et al.* 1974).

Al, incubated for 2 h in the salivary gland cells of the black fly Simulium vittatum, gained intracellular access and bound to chromatin with subsequent inhibition of ecdysterone induced "puffing" in 7 out of 9 chromosomal sites (Sanderson *et al.* 1982). Further support for Al localisation on DNA was provided by Dyrssen *et al.* (1987) who used both potentiometric titration and dialysis to show that *in vitro* Al ion binds to DNA obtained from calf thymus. It has been demonstrated that Al has cytotoxic effects on the the root-tip of Allium sativum, with mitotic depression which is associated with decreased DNA synthesis (Roy *et al.* 1989). Thus, there would seem to be a consistent effect of Al in attaching to DNA across a broad spectrum of biological systems.

It has been suggested that Al might complex irreversibly to polyphosphates of chromatid DNA in the cell nucleus (MacDonald and Martin 1988). In support of this, the nucleus has been noted to have the highest phosphate density of any cellular

organelle due to DNA and RNA phosphate content; concentrations of phosphate are 2,10, and 50 mmol/L in plasma, cytosol and the nucleus respectively (Lukiw *et al.* 1989).

1.3.2. **DNA strand breaks and nucleoids**

Nucleoids may be described as DNA in supercoiled form, present as protein-depleted nuclei, formed by cell lysis in the presence of detergent and high salt (Cook and Brazell 1976; Olive *et al.* 1986). Nucleoids lack histones but retain DNA organisation as a series of repetitive loops in a supercoiled conformation (Vaughan *et al.* 1990). Toxic damage to DNA leads to strand breaks which allow the relaxation of DNA superhelical structures with a consequent expansion of nucleoid size (Johnstone 1984). Assessment of nucleoid size does not give an absolute measure of the number of DNA strand breaks but rather indicates the relative change in size between two samples (Lipetz *et al.* 1982). The value of assessing supercoiled DNA damage is that it provides information both functionally (as it is the basic repeating unit of DNA replication), and in terms of structure (as it is physically defined) (Vaughan *et al.* 1990).

Nucleoid size can be assessed in various ways. Early techniques have used sucrose gradient sedimentation, where the larger nucleoids migrate more slowly than controls and are represented as a separate band in a test tube (Cook and Brazell 1976). This technique is influenced by viscosity,

particle density and structure as well as size. Other techniques have involved the use of DNA elution from filters, which requires bulk samples of cells (Vaughan *et al.* 1990).

A more recent method of measuring nucleoid size involves using a flow cytometer (FCM), such as the Epics 753 Fluorescence Activated Cell Sorter used in this work. The increase in nucleoid size presents a greater area for laser interaction, leading to an increase in light scatter being found in the forward angle light scatter (FALS) detector and which has been empirically related to particle size (Mullaney and Dean 1970; Loken and Stall 1982). Compared with the sucrose gradient sedimentation technique which represents pooled data gathered from a band of nucleoid derived material in a test-tube, the flow cytometer produces data individually gathered from thousands of nucleoids (Milner *et al.* 1987).

1.4. Flow cytometer

A flow cytometer (FCM) uses techniques of fairly recent origin which incorporate methodologies from laser technology, fluidics, computer science, and various fluorochrome and biochemical techniques. Such instrumentation allows for rapid quantitative analysis of cells or particles, as they pass singly through a laser beam, from which data are obtained and multivariate histograms can be derived (Carter and Meyer 1990).

1.4.1. Flow cytometer principles

The basic components (Figure 6) of a FCM are as follows (Carter and Meyer 1990):

a) Suitably prepared particles are injected into the flow chamber, where they enter a fast-moving liquid ("sheath fluid") which by a process of hydrodynamic focussing forces the individual particles into a single file.

b) The particles then pass individually across a laser beam where various interactions may occur. These changes are noted by photomultiplier tubes (PMTs) which are connected to computer data acquisition systems.

1.4.2. Flow cytometer parameters

The instrument is complex and capable of diverse functions. Only the parameters used in this work viz particle count; fluorescence; and forward light scatter will be discussed.

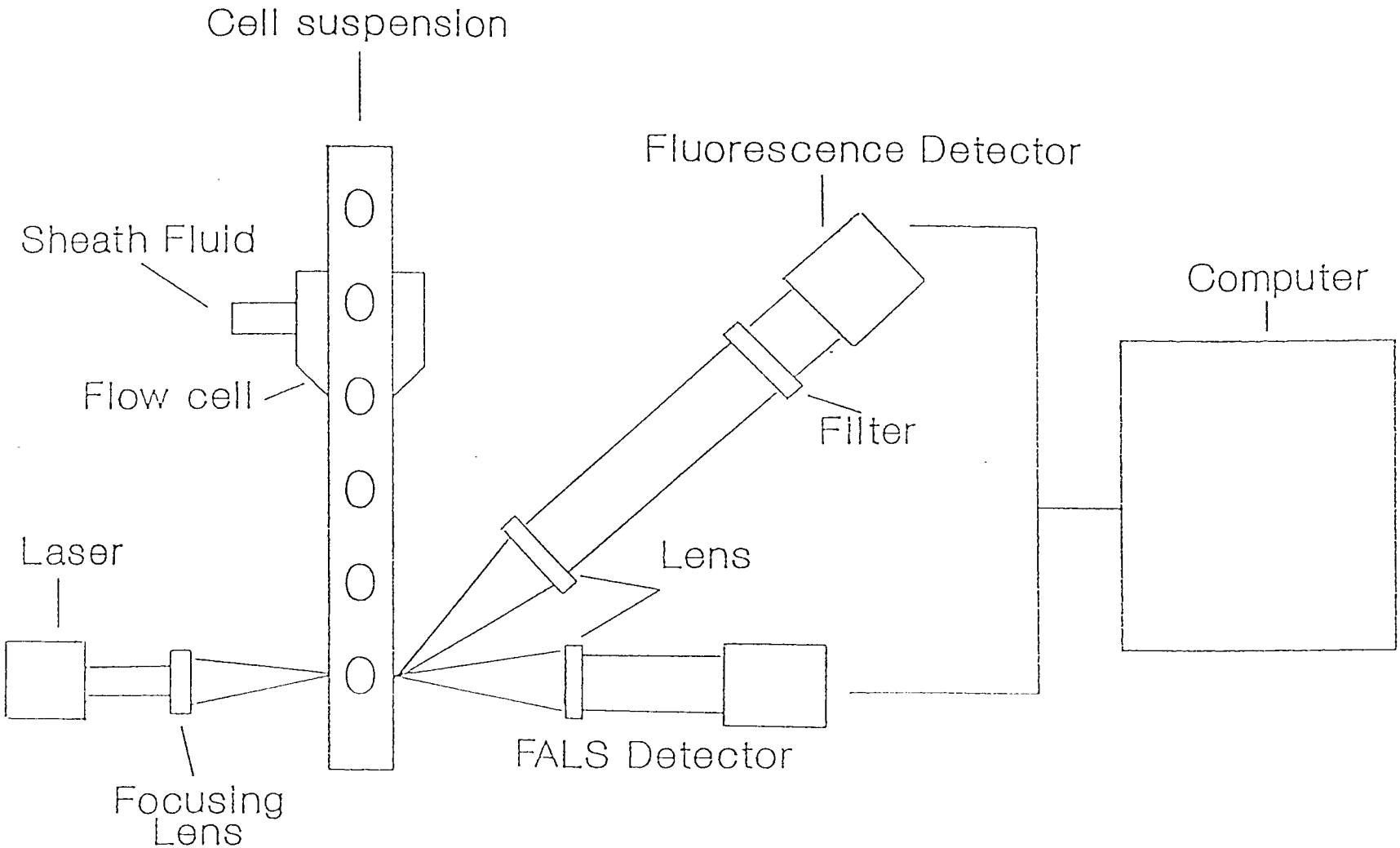

FIGURE 6: The operative components of the flow cytometer.

a) Particle count.

The passage of particles through the laser beam generates a signal which allows for their counting. Thus the number of particles processed can be precisely quantified.

b) Fluorescence.

Various fluorescent substances, such as ethidium bromide (EB) intercalate with double-stranded DNA. Their fluorescence emission on laser stimulation allows for the identification of DNA in the particles. Thus, by using this parameter other extraneous particulate matter which does not fluoresce (and therefore does not contain DNA) can be excluded from analysis. The use of EB in assessing nucleoid size is also of value as the compaction of DNA supercoils caused by the EB further enhances the sensitivity of this measurement (Vaughan *et al.* 1990). The same EB concentration was used in all our experiments to make this effect a constant one.

c) Forward angle light scatter (FALS).

The principle of light scatter used in FCM is analogous to the dark field illumination technique used in conventional microscopy which recognises cells as a result of their light scattering properties. As a particle passes through the laser beam, light is scattered from the particle in various directions. The light scattered in the forward direction (ie

in the direction the laser beam light is travelling) is caused mostly by diffraction. Particles of different sizes generate different scatter patterns. The total amount of light scattered in the forward direction (FALS) is directly related to particle size (Mullaney and Dean 1970; Loken and Stall 1982).

2. METHODS

2.1. Establishment of cell line

A pig kidney adherent epithelial cell line (LLC-PK1) was obtained from a cell repository (American Tissue Culture Collection Cat No CL 101). This is a homogenous cell line with characteristics of proximal tubular cells (Hull *et al.* 1976; Cantiello *et al.* 1986; Holohan *et al.* 1988). The cells were grown in M199 media (Cat No PO6, Highveld Biological (Pty) Ltd, Johannesburg) supplemented with 3% fetal calf serum. As Al binds to proteins and phosphate it was necessary for the experiments to quantify cell growth characteristics both in serum free (-S) and phosphate free (-Pi) media (constituents listed on page 245a). The ability of these cells to grow in serum-free conditions has been previously established (Hull *et al.* 1976).

2.2. DNA synthesis

DNA synthesis was measured using ^{3}H-TdR based on a previously

described method (Bradley 1980).

LLC-PK1 cells were plated at a concentration of 50 000 cells/100µl volume of (-Pi-S) media in 96 well plates. 100µl of A1 containing medium, to produce a final arbitrary dose range of 0.01-8 mM, or pH matched (-Pi-S) media as controls, was added to each well. Tritiated thymidine (specific activity 20 Ci/mmol, New England Nuclear) was added to each well at the beginning of the experiment at a concentration of 2 µCi in 50 µl of media.

At the end of the experiment EDTA-trypsin was added and the cells collected and washed three times with water using a cell harvester. The filter papers containing the harvested cells were dried, placed in Aquagel (Chemlab Ltd) and measured in a Tri Carb 300 Scintillation Counter (Packard Instrument Co Inc., Illinois, USA). The ^{3}H-TdR content of the cells was assayed at 9, 12, 24, 48 and 72 hours, at least in triplicate. The results obtained represent the cumulative incorporation of ^{3}H-TdR into the cells.

2.3. DNA strand breaks

Nucleoid size as a measure of DNA strand breaks was analyzed based on a previously described method (Vaughan *et al.* 1990).

LLC-PK1 cells in (-Pi-S) media were filtered through a 72µ polypropylene mesh to produce a single cell suspension. The cells were then distributed into Eppendorf tubes in a volume

of 0.5 ml cell suspension/tube. Al, made up in (-Pi-S) media, to produce a final arbitrary dose range of 0.001-32 mM (or pH matched media as controls) was then added in a volume of 0.5 ml at 5 min intervals (in order to standardise the timing of each tube precisely) so that the final tube volume was 1 ml.

The Eppendorf tubes were incubated for 90 min in a CO_2 incubator (Model 3614, Forma Scientific Inc., Marietta, Ohio, USA). The cells were washed in (-Pi-S) media twice and to the cell pellet was added 1 ml ice cold lysis buffer (containing 2M sodium chloride, 10 mM TRIS, 10 mM disodium EDTA, and 0.5% Triton-X 100) in order to produce nucleoids. After being maintained at 0 °C for 30 min 0.1 ml ethidium bromide (made up in water at a concentration of 0.5 mg/ml) was added. Tubes were then kept on ice for 90 s to allow for dye equilibration and then read on the flow cytometer.

The nucleoid size was analyzed in a flow cytometer (FCM) ie the Epics 753 Fluorescence Activated Cell Sorter (Coulter, Hialeah, Florida, USA) interfaced with an MDADSII data acquisition and analysis system.

The FCM operating parameters were as follows. Argon ion laser line at 488 nm (250 mW) was used for excitation. The particles were processed through a 150µ flow cell tip at a flow rate less than 400 particles/second. The data on forward angle light scatter (FALS) and fluorescence emission (using a 610 nm band pass filter) were collected, amplified and scaled to

generate multiparameter histograms. The fluorescence emission was gated to exclude non-DNA particles, and subsequently analysis gates were set on FALS to determine the mean channel for each specimen. After an equilibration period of 20 s a minimum of 15 000 particles were analyzed for each histogram generated.

3. RESULTS

3.1. Establishment of cell line

Figure 7 demonstrates the growth of the cells in (-S) and (-Pi) conditions up to 72 hours. Cell proliferation continues to take place in (-S-Pi) conditions.

3.2. DNA synthesis

The ^{3}H-TdR assay is a sensitive indicator of thymidine incorporation and indicates the rate of DNA synthesis. Figure 8 shows that at 0.01 mM Al no difference between the Al and control groups could be observed up to 72h. However, beginning at 0.05 mM Al the rate of thymidine incorporation by the cells begins to decrease. From a concentration of 0.05 to 1.0 mM Al, thymidine incorporation was decreased in the Al group. At the 2.0-8.0 mM Al range there was virtually no incorporation of ^{3}H-TdR in the Al group, while in the pH matched control group incorporation continued.

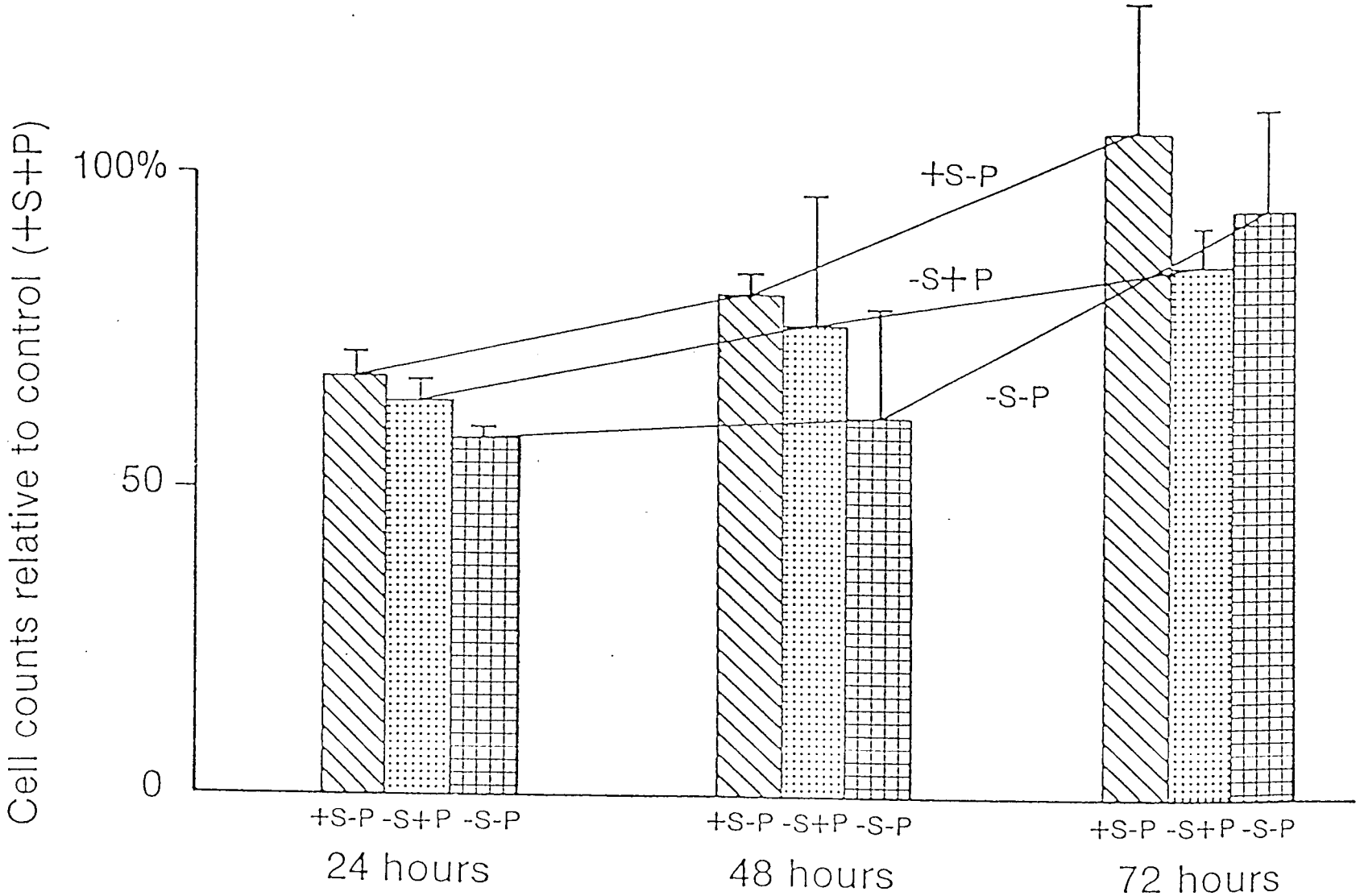

FIGURE 7: Cell growth of LLC–PK1 in serum (S) and phosphate (P) free conditions for 72 h.

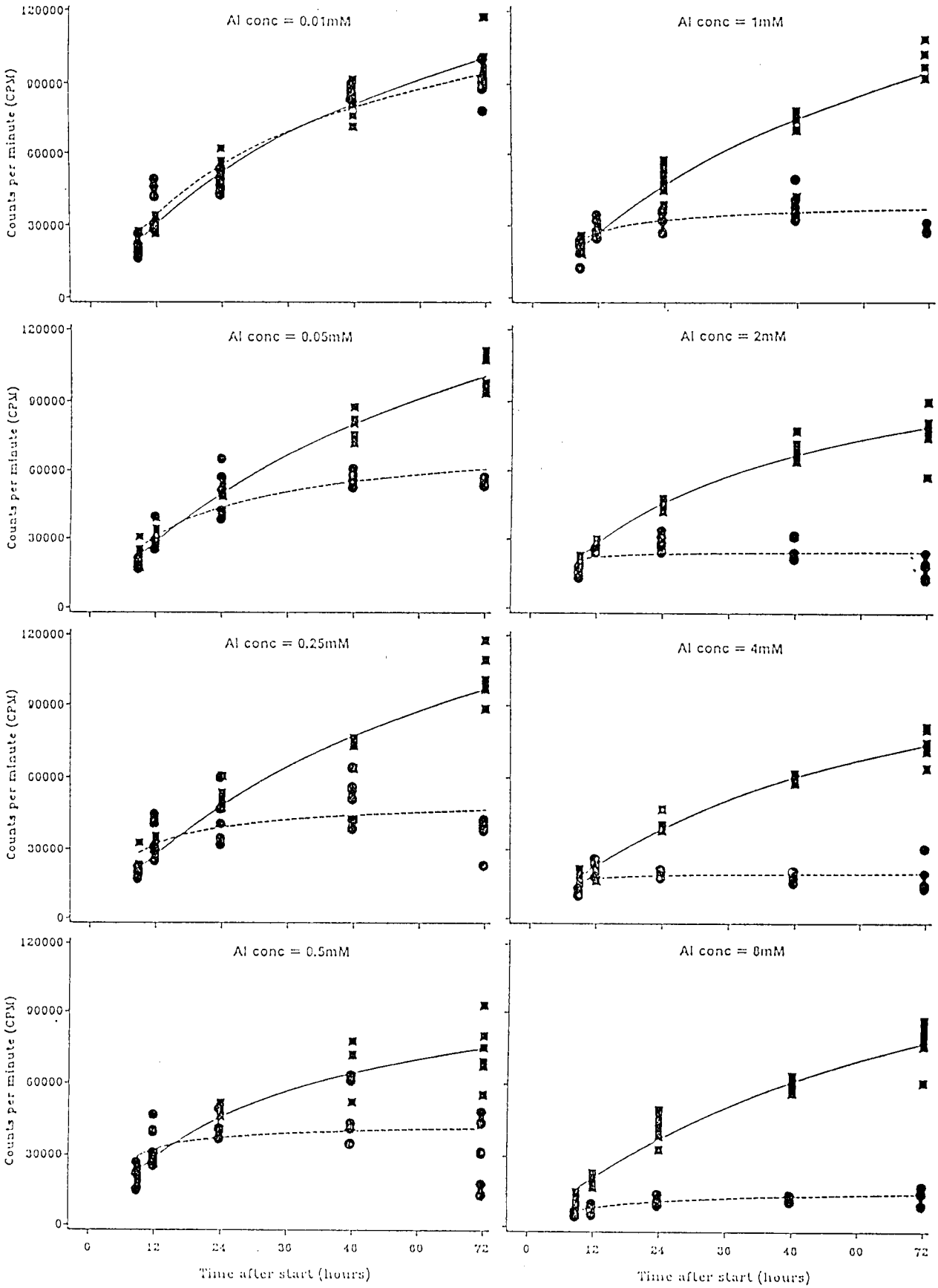

FIGURE 8: Cumulative incorporation of 3H-TdR in cultured kidney cells from 9-72 hours. Al treated and control groups are shown by dashed and continuous lines, respectively.

3.3. DNA strand breaks

Figure 9 demonstrates the mean forward light scatter channel (representing nucleoid size) of each Al sample as a percentage of its pH matched control. There was little if any increase in nucleoid size compared with controls from concentrations 0.001-0.05 mM. At concentrations greater than 0.05 mM Al there was an increase in nucleoid size relative to controls.

The data were fitted using a modified (Dr I Havlik, personal communication) Michaelis-Menten equation

$$S = \frac{S_{max} \cdot C}{Q_{so} + C} + S_o$$

where S represents relative nucleoid size compared to controls;

Subscripts S_{max} and S_o represent the maximal nucleoid size, and the extrapolated nucleoid size at zero Al concentration, respectively;

Q_{so} is the value of Al concentration (in mM) when half the maximal effect (S_{max}) is achieved.

and, C is the concentration of Al in mM.

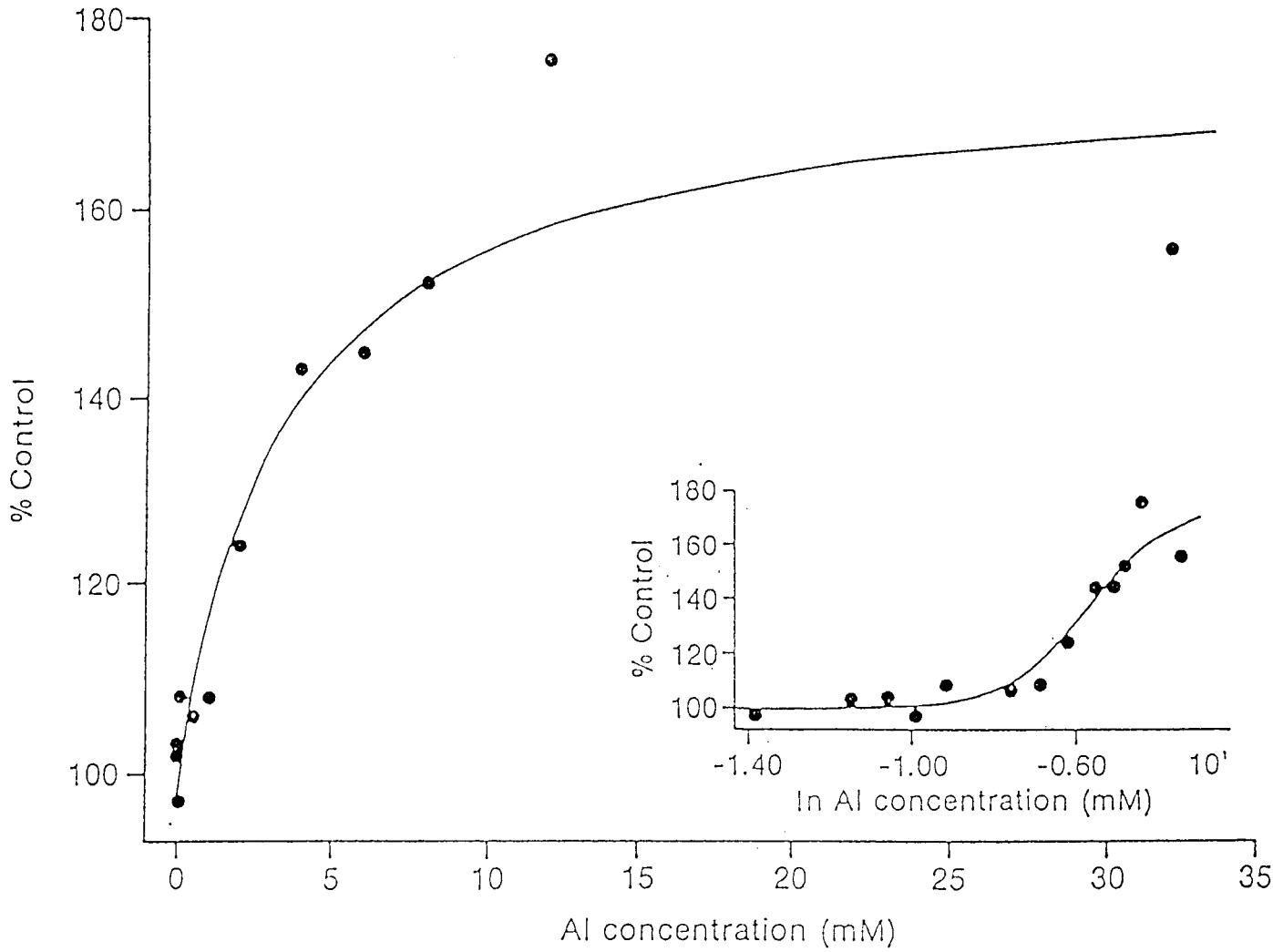

FIGURE 9: Nucleoid size measured by the mean gate of the forward light scatter at various Al concentrations (0.001–32 mM), expressed as a percentages of pH–matched controls. The insert represents the same data logarithmically transformed.

For clarity, the data were also logarithmically transformed, and are displayed as an insert in the main graph. The data were fitted using Enzfitter software (Biosoft, Cambridge, UK), a Marquart algorithm (Leatherbarrow 1987) and the reduced chi-square as the iterative parameter. The maximal nucleoid size (S_{max}) was 76 (SE $\pm$ 9.6) % greater than the control size. The Al concentration at which the nucleoid size was half the maximal size (Q_{50}) was 3.44 (SE $\pm$ 1.50) mM. Using the fitted data the origin of the curve representing the extrapolated value (S_o) was 99 (SE $\pm$ 3.5)% .

Figure 10 a-m) illustrates a series of 3-D histograms of Al treated nucleoids and their pH matched controls. No discernible change could be noted between the Al and control samples at low concentrations, whereas obvious changes could be noted between the treated and control samples at higher concentrations (Figure 10). The Al treated 3-D histograms at higher concentrations (Figure 10) illustrate the increases in particle size shown in Figure 9.

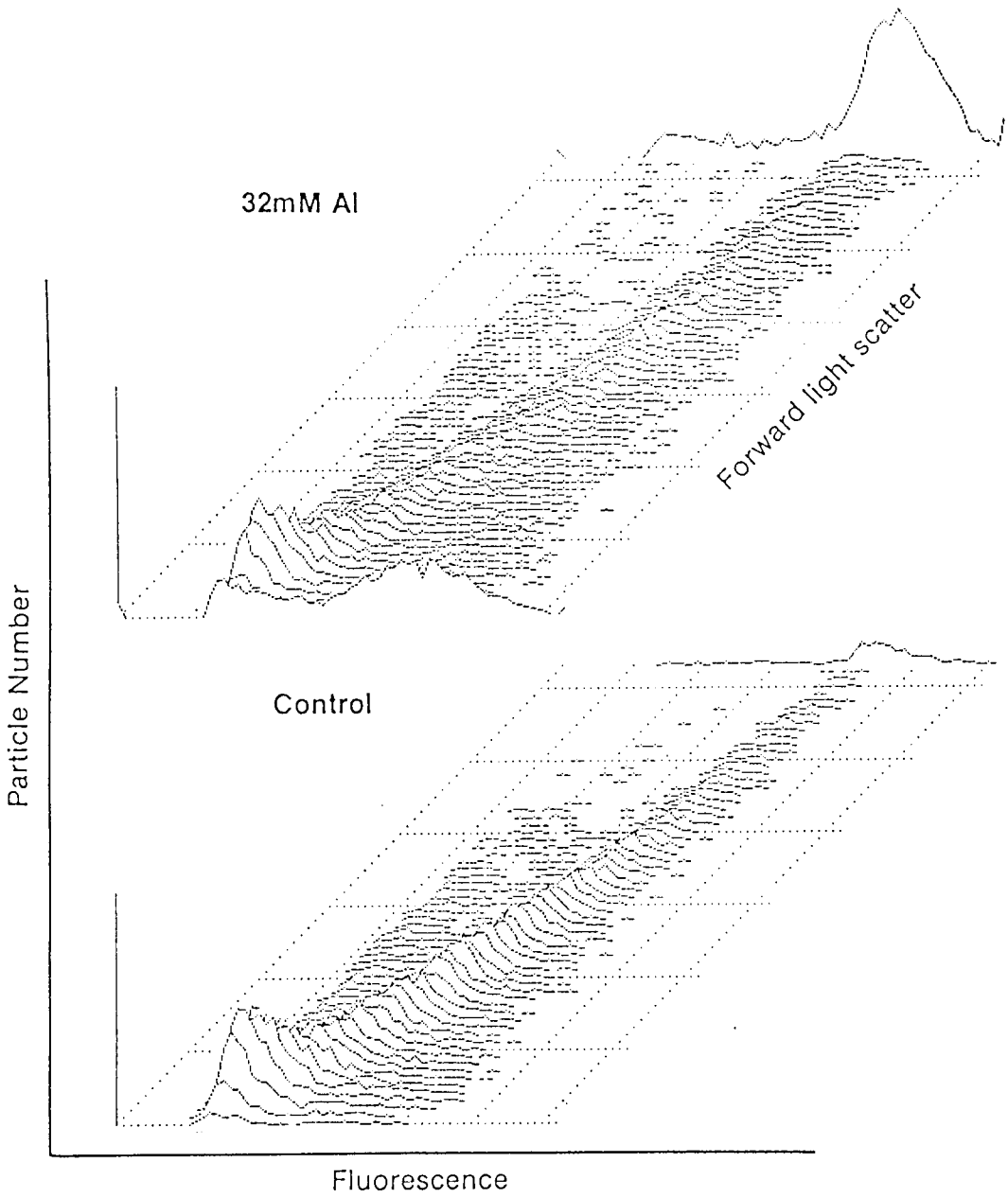

FIGURE 10a: 3-D histograms of 32 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

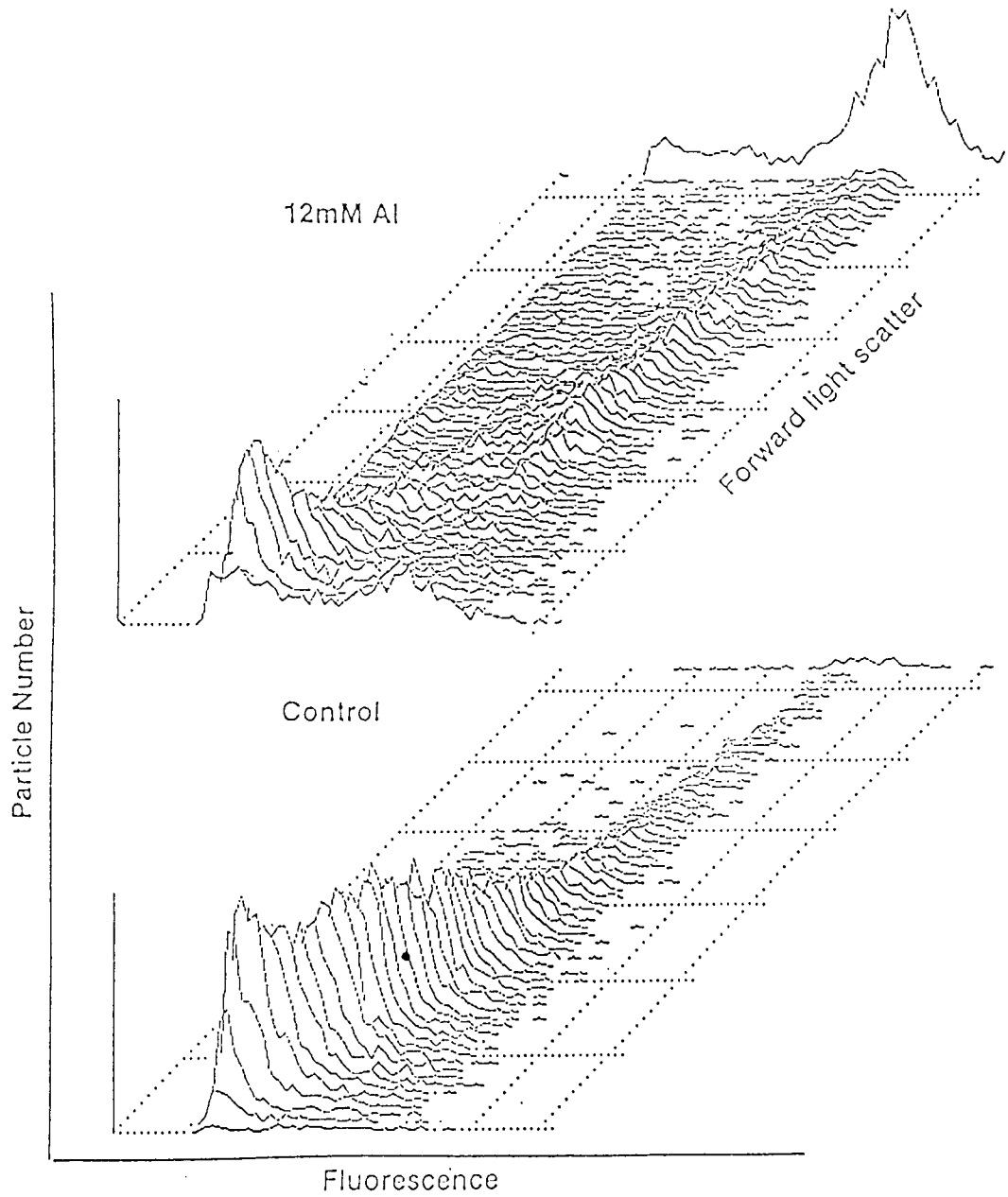

FIGURE 10b: 3-D histograms of 12 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

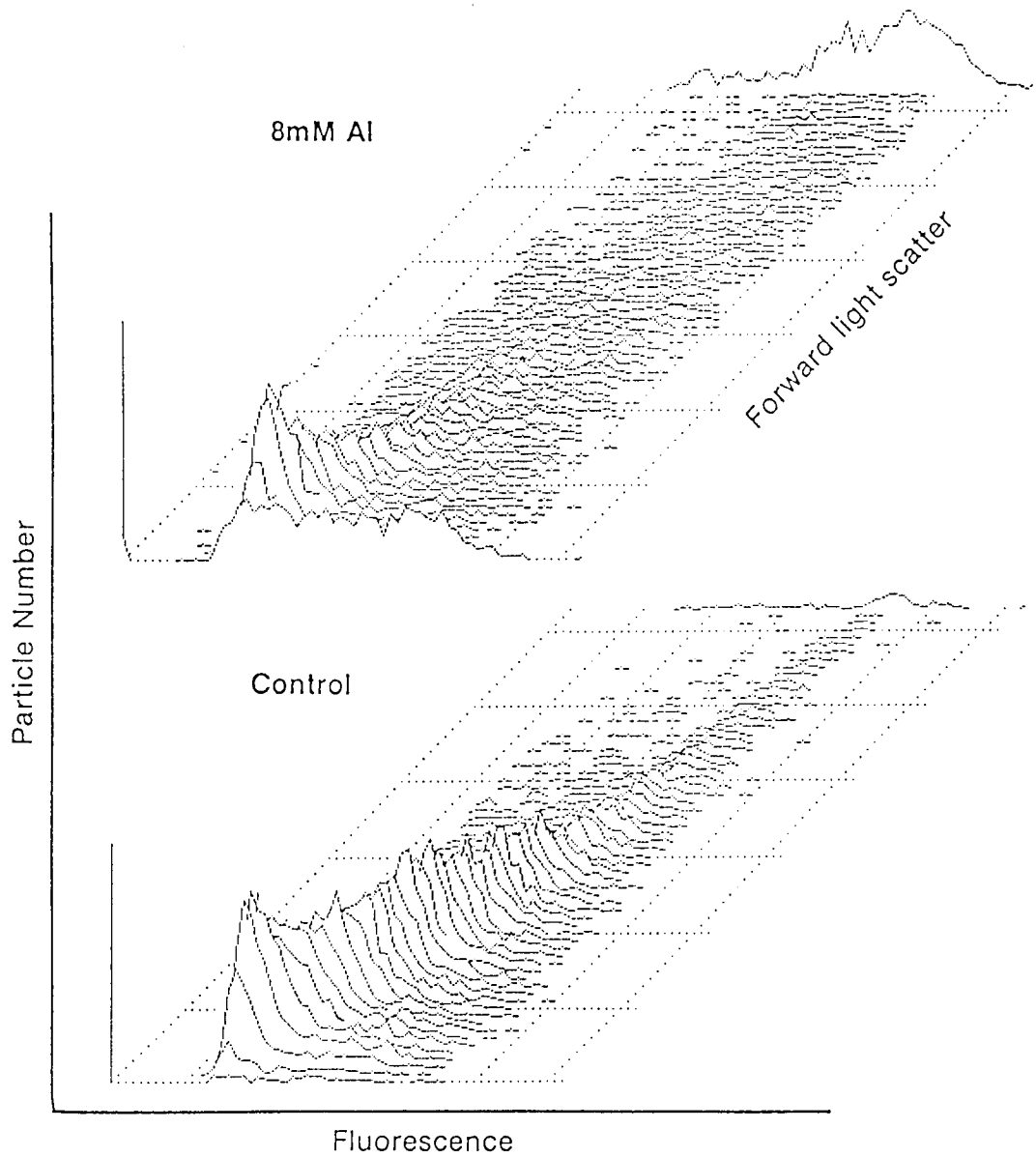

FIGURE 10c: 3–D histograms of 8 mM Al nucleoids and control. The height of the peaks on the Y–axis demonstrates the particle number; the X–axis demonstrates the range of fluorescence of DNA; and the Z–axis represents forward light scatter with the particle size increasing into the distance.

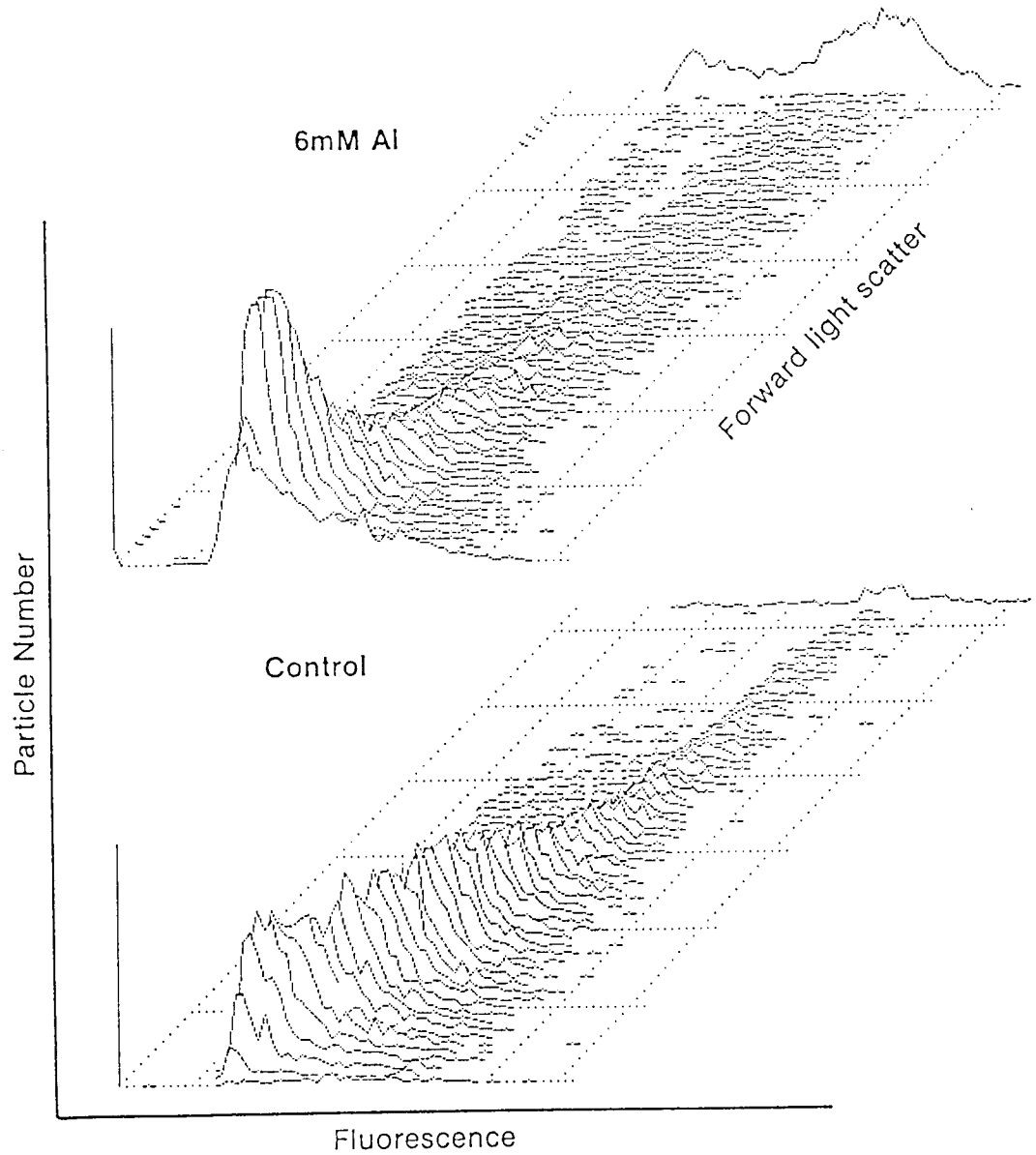

FIGURE 10d: 3-D histograms of 6 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

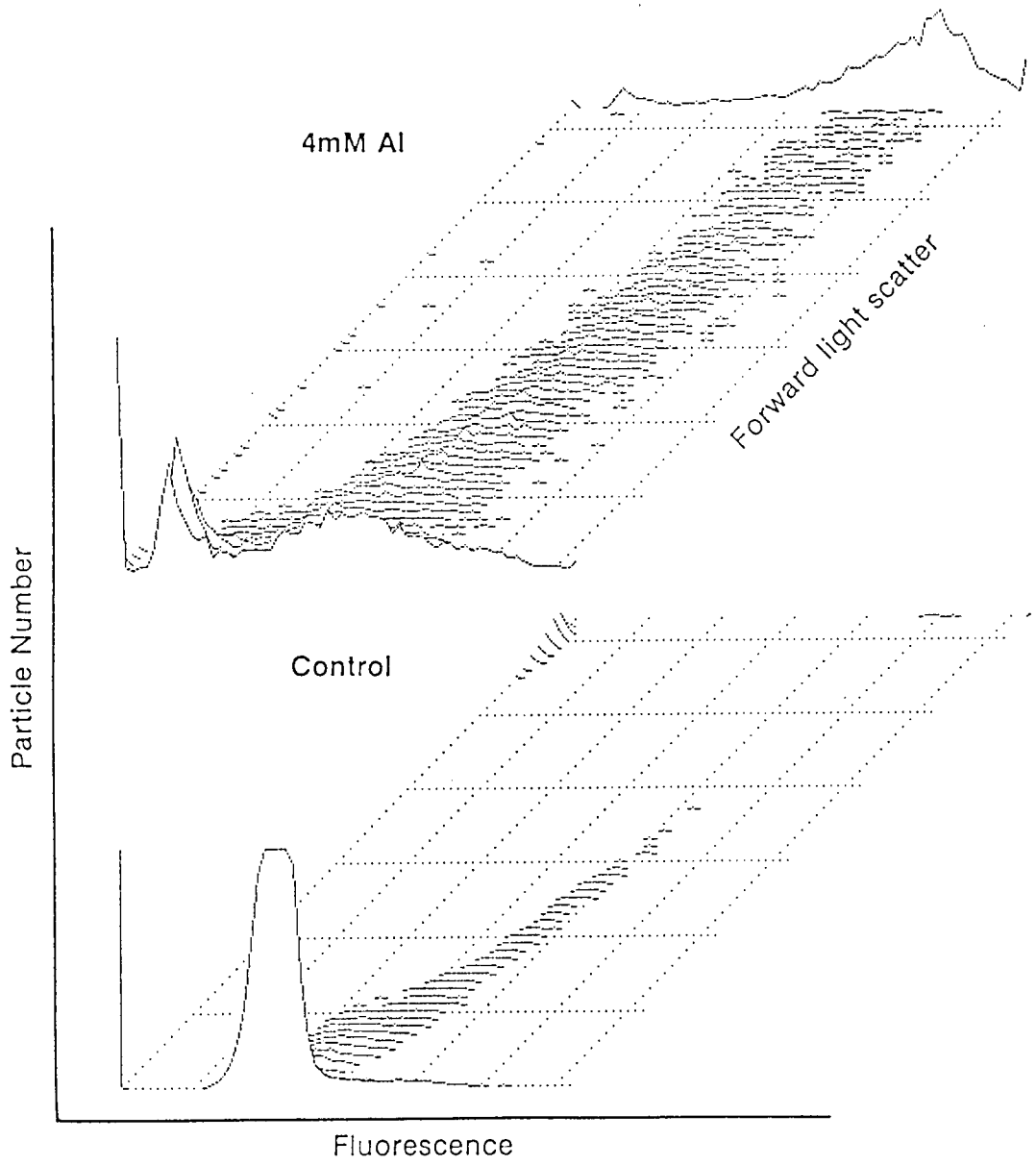

FIGURE 10e: 3-D histograms of 4 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

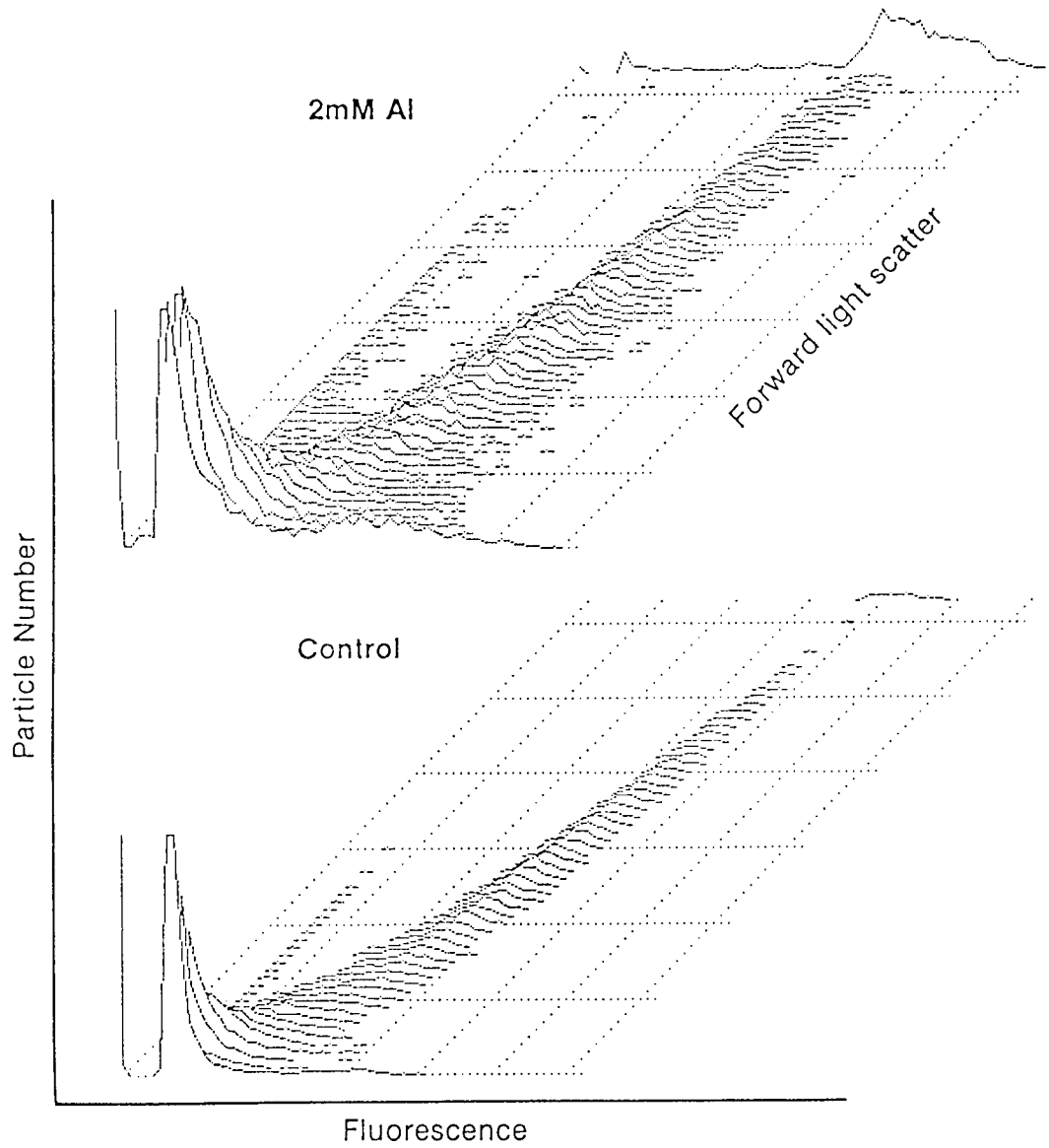

FIGURE 10f: 3-D histograms of 2 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

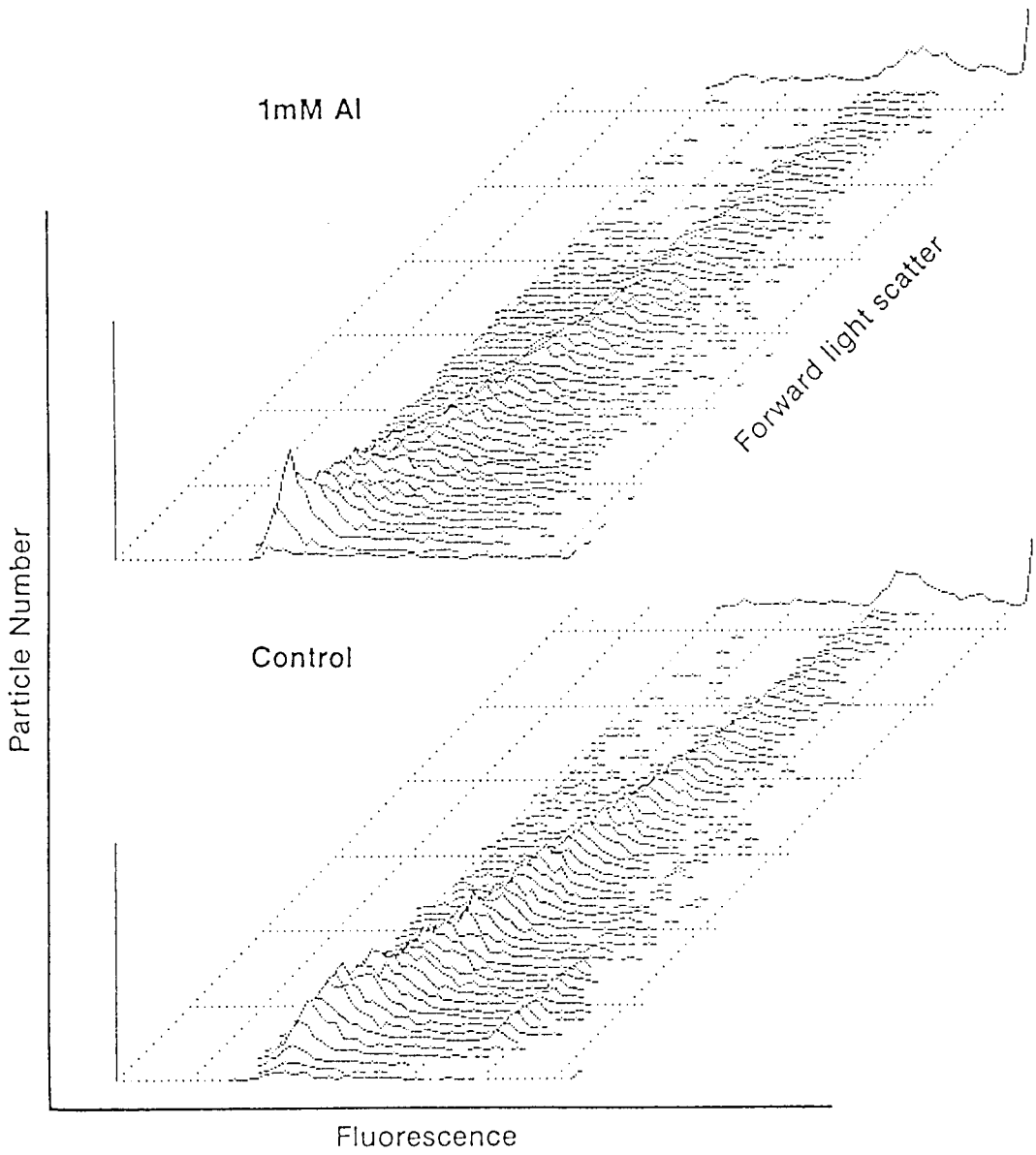

FIGURE 10g: 3-D histograms of 1 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

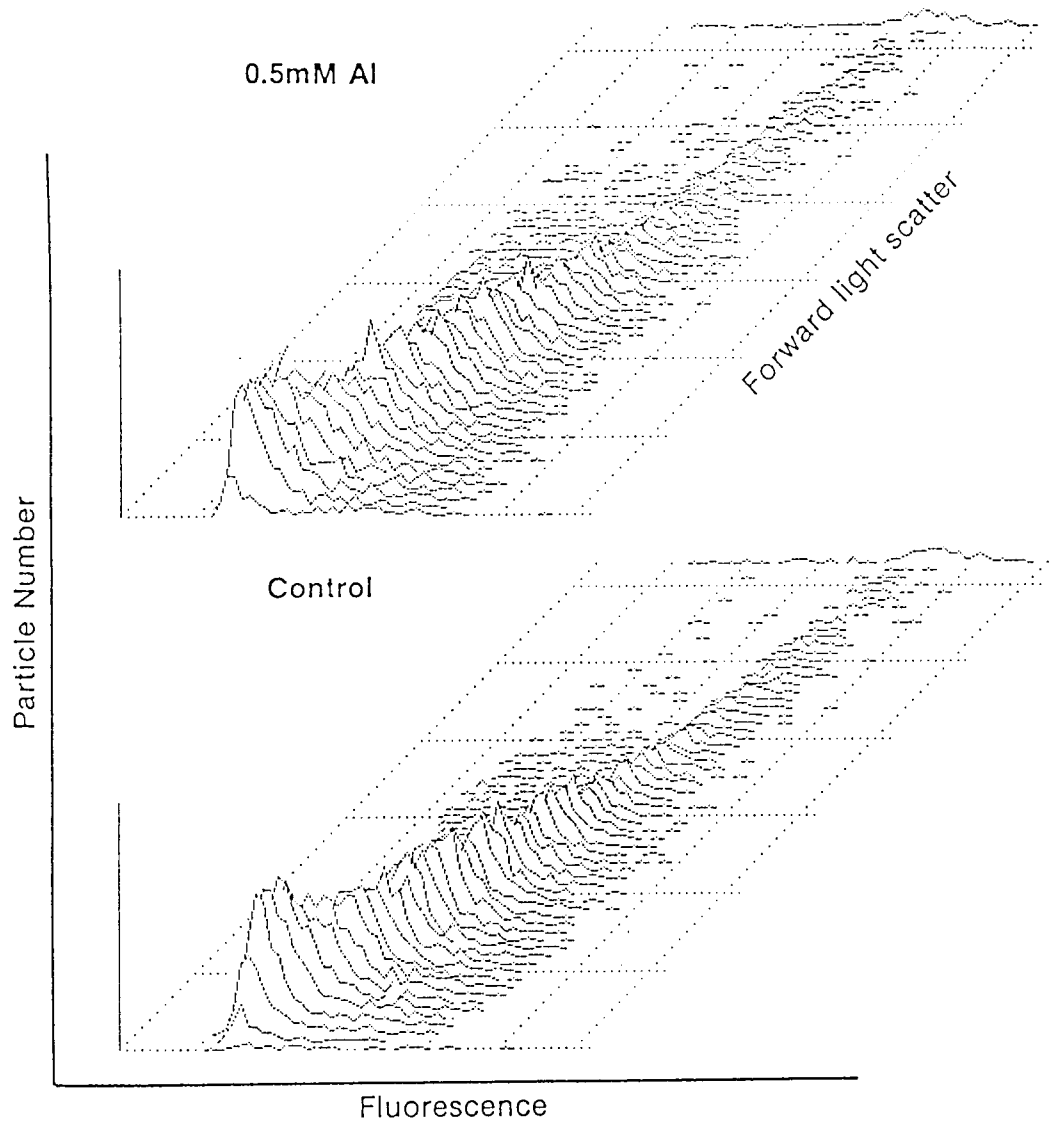

FIGURE 10h: 3-D histograms of 0.5 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

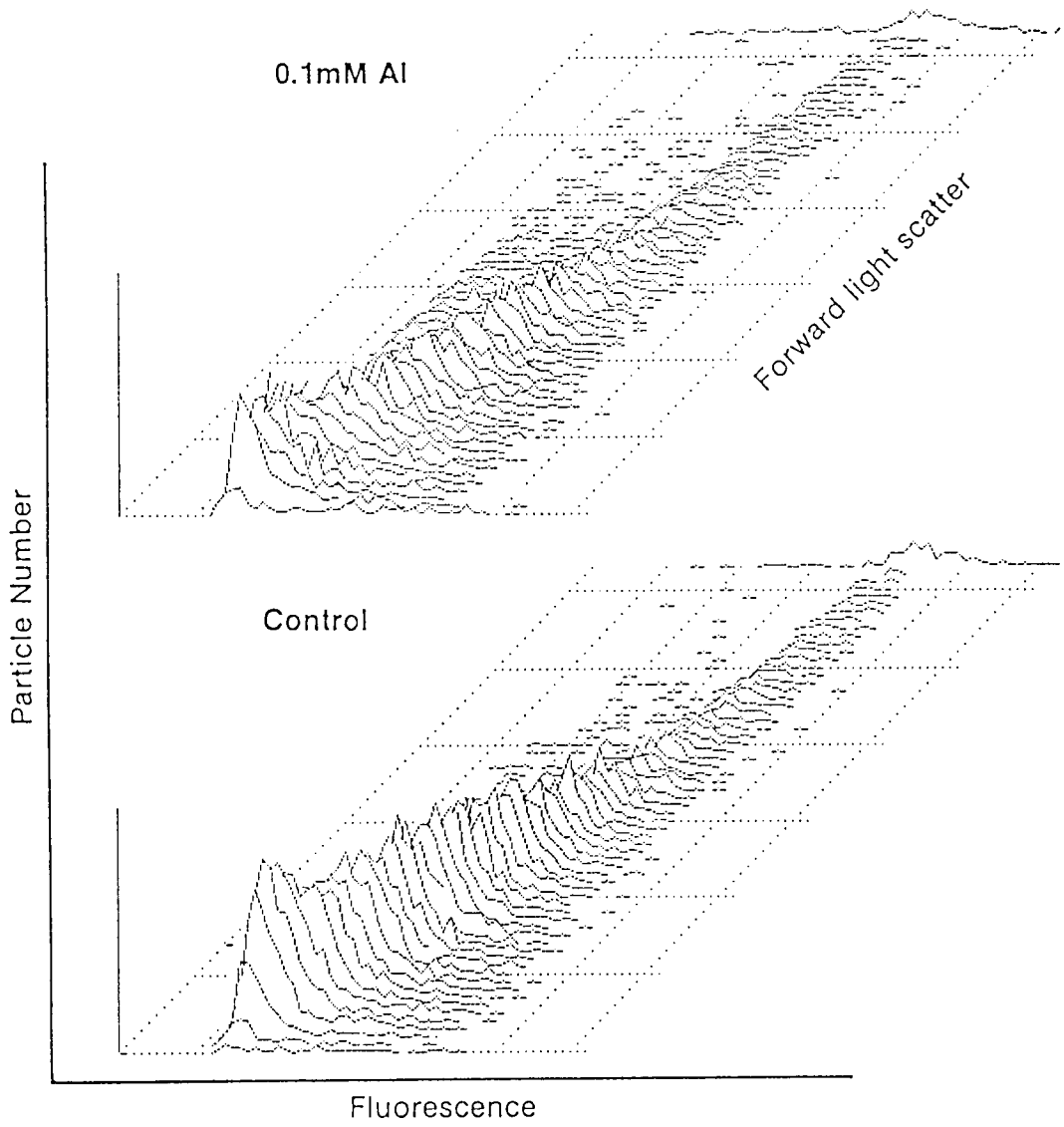

FIGURE 10I: 3-D histograms of 0.1 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

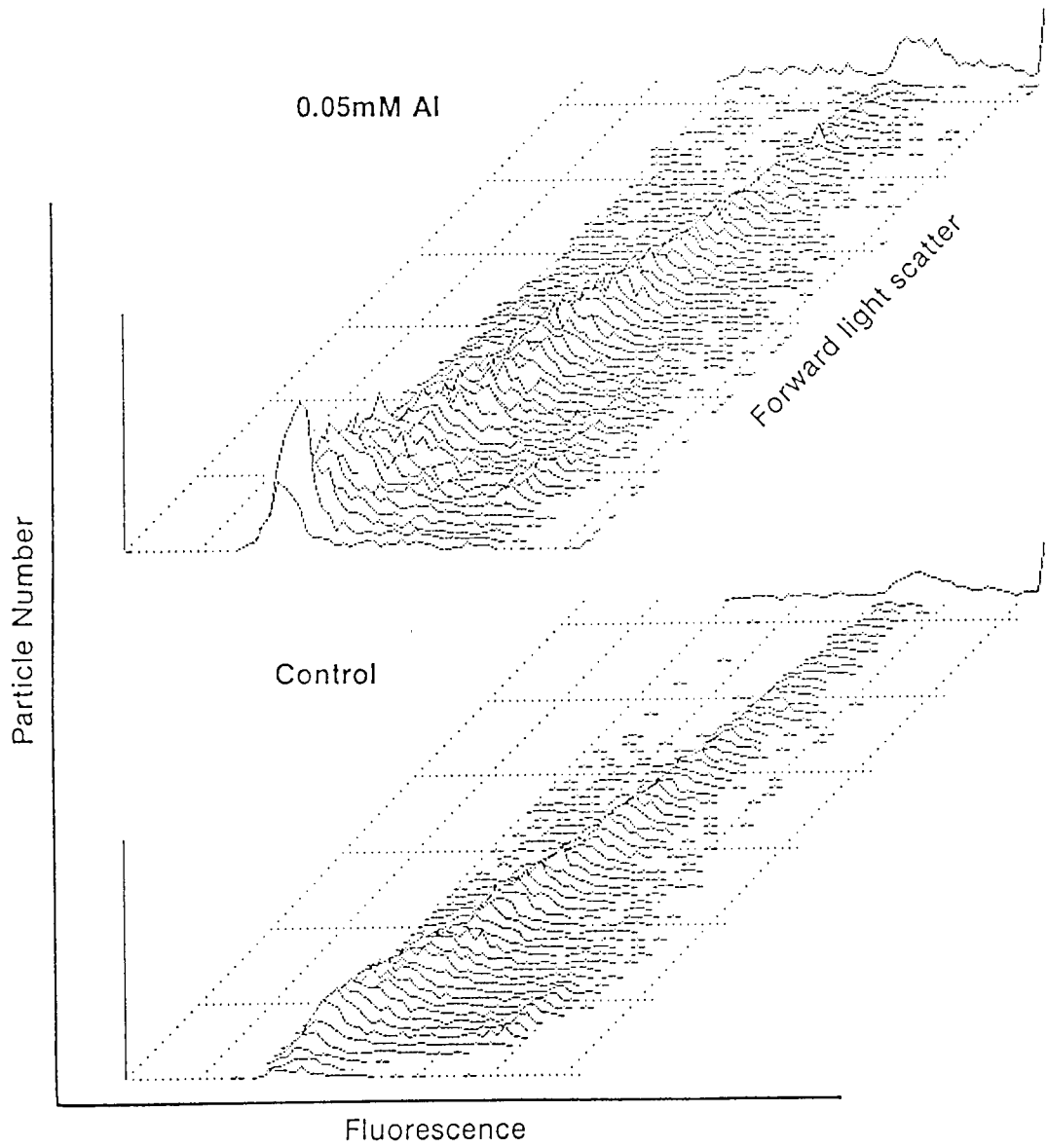

FIGURE 10j: 3-D histograms of 0.05 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

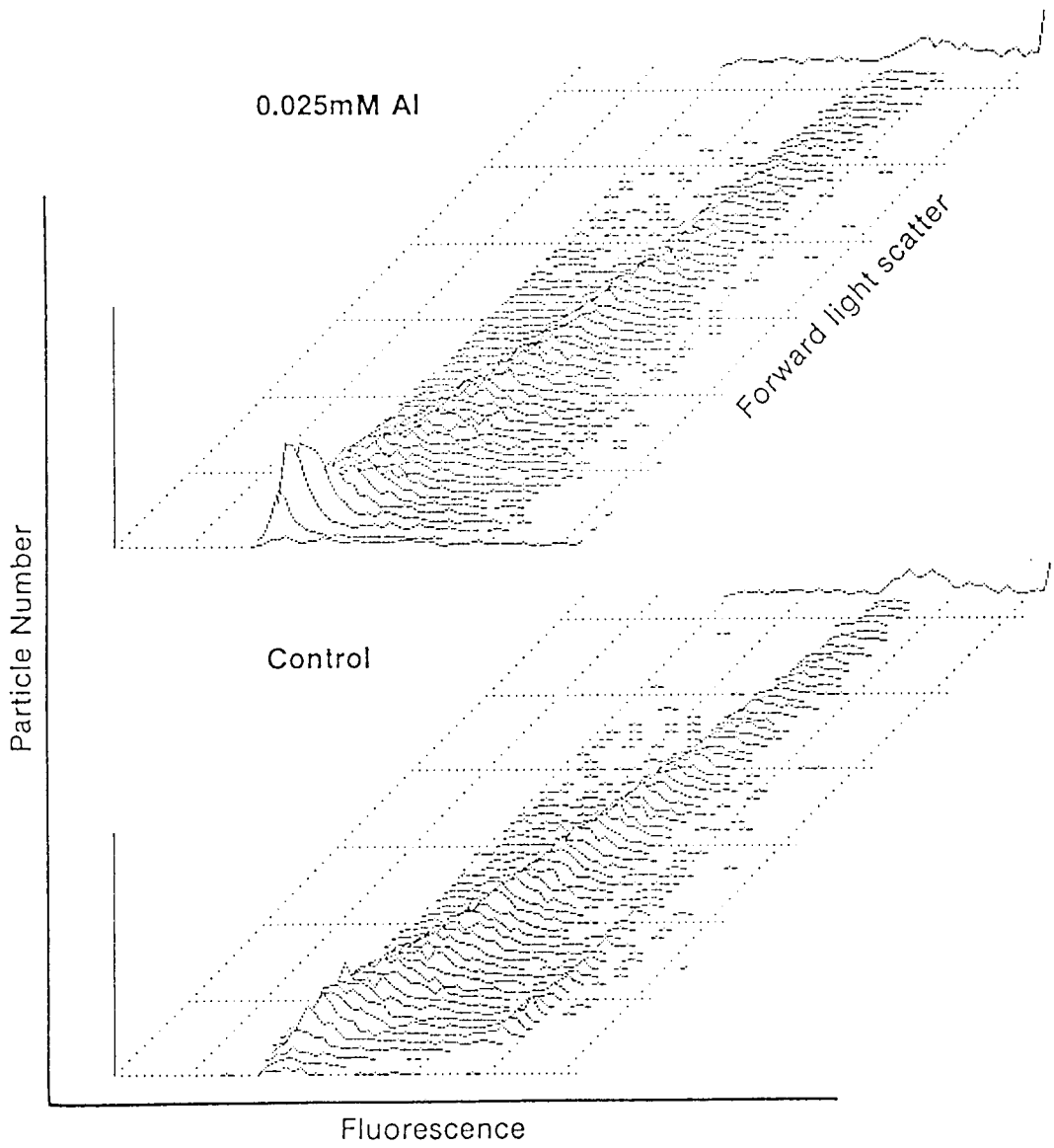

FIGURE 10k: 3-D histograms of 0.025 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

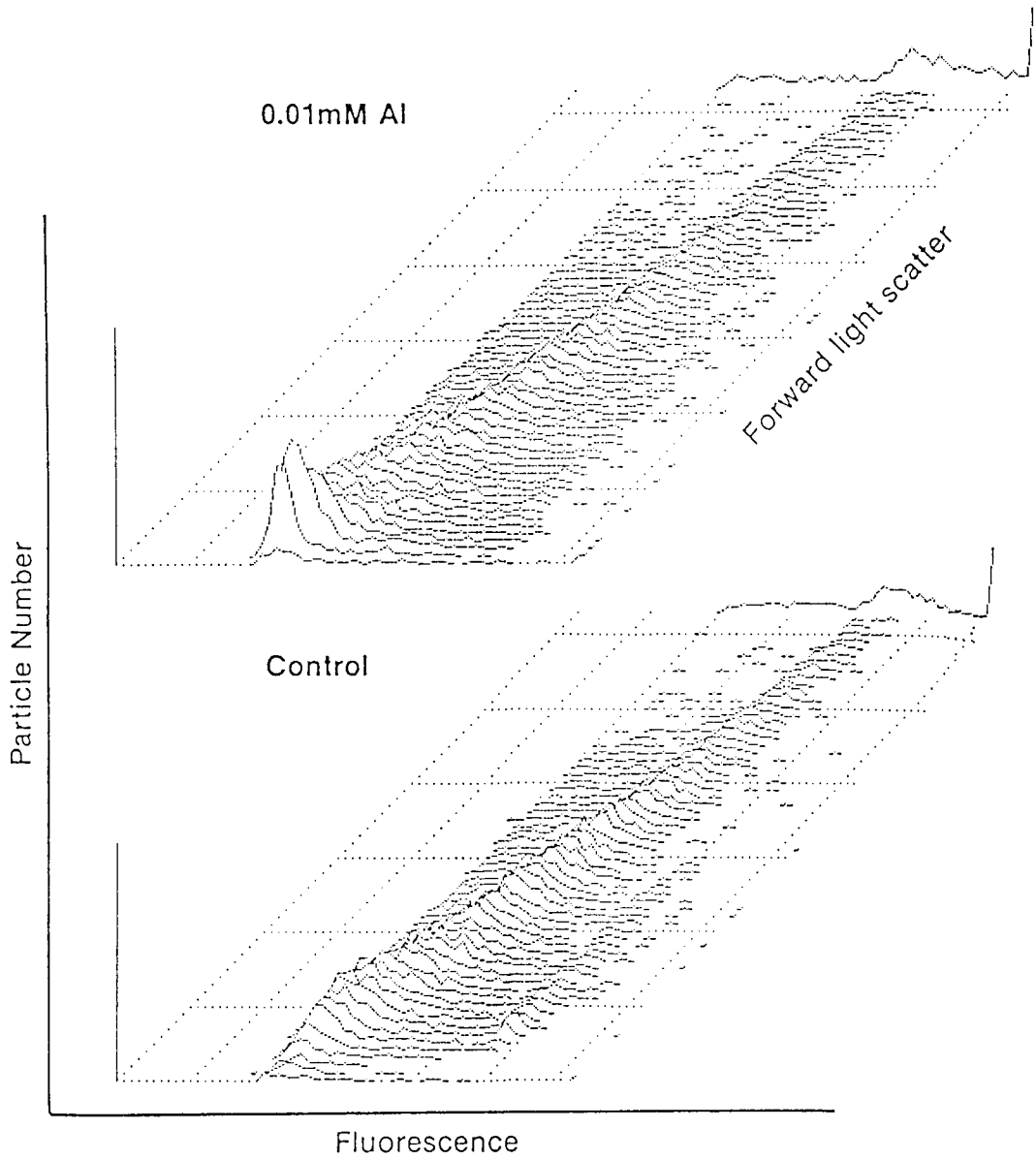

FIGURE 10I: 3-D histograms of 0.01 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

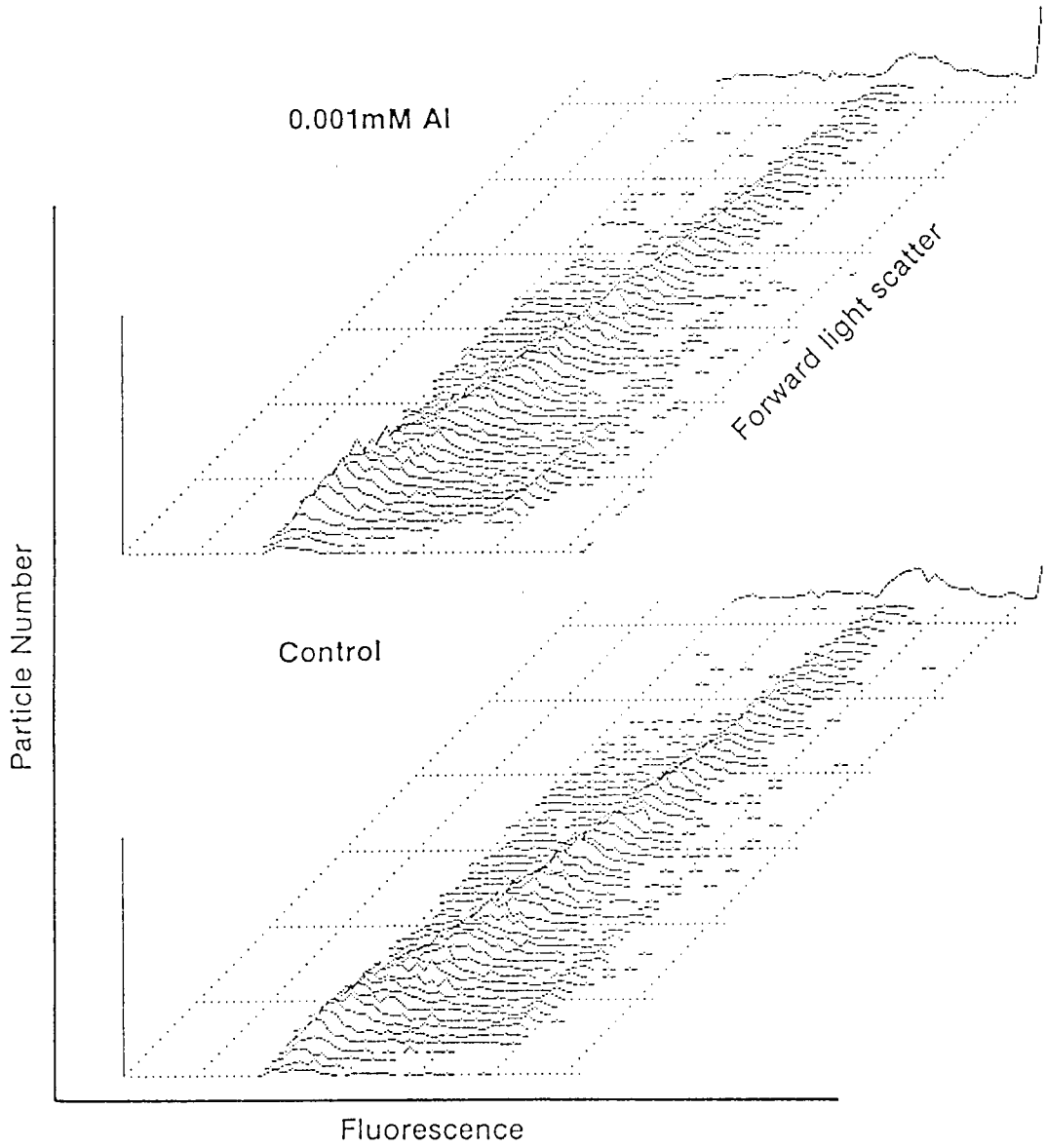

FIGURE 10m : 3-D histograms of 0.001 mM Al nucleoids and control. The height of the peaks on the Y-axis demonstrates the particle number; the X-axis demonstrates the range of fluorescence of DNA; and the Z-axis represents forward light scatter with the particle size increasing into the distance.

4. DISCUSSION

4.1. Discussion of literature

The role of kidney disease in causing Al toxicity has been previously described and relates to impaired excretion of the element (Monteagudo *et al.* 1988; Monteagudo *et al.* 1989). The converse role of Al toxicity in causing renal disease has not been fully explored. Al toxicity could possibly play a role in damaging kidney tissue and further aggravate pre-existing renal disease. The role of Al as a toxic agent in various cell types other than kidney, such as brain, haemopoietic cells, and bone is well known.

Previous work has demonstrated tissue accumulation, as well as functional effects, of Al on the kidney. Al has been shown to accumulate in the kidney (Henry *et al.* 1984; de Galle 1981). Kidney function has been found to be impaired in the presence of Al, both in terms of concentrating ability (Braunlich *et al.* 1986), as well as creatinine clearance (Henry *et al.* 1984). However, Cacini and Yokel (1988) did not find Al at concentrations of 1 mM over 2 h to be toxic in incubated slices of rabbit renal cortex.

It is suggested that some of the cellular and molecular mechanisms of Al action may explain its toxicity. Al has been shown to bind to chromosomal material (de Boni 1974); to

interfere with DNA and RNA (Siegel 1985); to inhibit ADP-ribosylation (Crapper McLachlan *et al.* 1983); to inhibit brain glycolysis (Lai and Blass 1984); to inhibit hydropteridine reductase in erythrocytes (Altmann *et al.* 1987); to inhibit calmodulin (Suhayda and Haug 1984); possibly to impair the inositol phosphate system (Birchall and Chappell 1988); to inhibit magnesium dependent enzymes (Macdonald and Martin 1988); and to interact with calcium channel blockers (Provan and Yokel 1988). The cellular site of toxic action of Al is obviously not clearly defined although DNA damage has clearly been implicated. Many of the above processes may interfere with DNA synthesis, or cause DNA strand breaks, by secondary mechanisms.

4.2. Discussion of experiments

These experiments were performed in serum and phosphate free (-S-Pi) conditions as both these substances are known to bind to Al and thus reduce its biological availability. LLC-PK1 cells have previously been shown to adapt to growth in serum free conditions (Hull *et al.* 1976). Cell growth continued for up to 72 hours in (-Pi-S) media (Figure 7). This growth in (-Pi-S) conditions was confirmed in the ^{3}H-TdR experiments which showed continued incorporation up to 72 hours in the controls.

In the first experiment, ^{3}H-TdR incorporation was used as a marker of continued DNA synthesis and subsequent cell proliferation. The data demonstrate that at an Al

concentration of 0.05 mM an effect on ^{3}H-TdR incorporation became more prominent in the later stages of the experiment. Little, if any, effect could be seen at 9 and 12 hours. At higher concentrations of Al, toxic effects were more prominent at earlier times in the experiment. It is possible to speculate that exposure to very much smaller doses of Al over years could exert a similar toxic effect.

The second experiment demonstrated the effect of Al on nucleoid size as a marker of DNA strand-breaks. The mean gate on the FALS detector, representing particle size for each Al concentration, as a percentage of controls is shown in Figure 9. At concentrations greater than 0.05 mM increases in particle size were noted with a calculated eventual maximal nucleoid size of 76 $\pm$ 9.6% greater than control (which is taken as 100%). This increase in nucleoid size, particularly in the dose dependent manner shown, is indicative of the effect of Al in causing DNA strand-breaks.

Figure 10 a-m) shows a series of 3-D histograms providing additional information on the nucleoid particles represented in Figure 9.

These 3-D histograms provide a pictorial representation of the number, degree of fluorescence, and size of the particles in question. Thus, they provide qualitative, rather than quantitative, information. Due to differences in the number of particles present in certain peaks the scaling was adjusted to

best represent that particular graph. At the "deepest" edge of the Z-axis, a so-called "horizon line" is evident in all the graphs. Peaks on this "horizon line" represent a summation of all particles larger than those represented in the histogram range of the Z-axis.

Inspection of the series of 3-D histograms illustrates the differences at higher Al concentrations where there appear to be greater numbers of larger particles compared with controls. At lower Al concentrations the differences between the treated and control groups are less marked.

The data found in this section represent the results of *in vitro* experiments. The concentrations of Al used *in vitro* were higher than those encountered in clinical practice. Higher concentrations were used due to the very short period of exposure *in vitro* (hours) compared with clinical exposure (often years). Moreover, despite the (-Pi-S) conditions of the experiment Al binds to substances, other than serum and phosphate, in *in vitro* conditions thus reducing biological availability and effect.

In the clinical setting, patients with renal failure often have blood levels of 200 µg/L or higher (Savory 1985). Blood levels of 1730 µg/L have been recorded (De Broe *et al.* 1984). However, the level of Al in the blood may bear little direct relationship to the concentration of Al at its cellular site of toxic action in the body.

This study of the toxic mechanisms of Al on kidney cells may be of importance firstly by increasing knowledge of clinical syndromes of Al toxicity and secondly by providing information on cell biology and molecular mechanisms. The process by which Al exerts an effect on DNA synthesis and DNA strand breaks is largely speculative at present but may involve more than one mechanism.

SECTION D

SUMMARY

This thesis has studied aspects of the interaction between aluminium and the kidney. The work has addressed two major issues.

Firstly, a site for aluminium excretion by the distal tubule of the pig kidney was identified. The role of the kidney in excreting Al is critical as has been demonstrated clinically. Possible avenues for further research into the mechanisms involved in such excretion, how these observations relate comparatively to other species, and the role of pharmacological agents in increasing this excretory process, are suggested by the findings.

Secondly, Al interacts with DNA causing diminished synthesis, and production of DNA strand-breaks, in a kidney cell system *in vitro*. This effect of Al on DNA throws light on the mechanisms of toxicity of the element in biological systems. How it is that aluminium exerts the effects on DNA that have been described in this thesis remain to be determined.

APPENDIX

DATA FROM SECTION B

INTRODUCTION TO APPENDIX

The Appendix deals with data from Section B. This introduction is inserted to facilitate the reading of this data.

SUB-INDEX OF APPENDIX PAGE

SPECIFIC APPENDIX ABBREVIATIONS

1. N/A - not available
2. N/D - non-detectable
3. S-F(I) - refers to interrupted stop-flow
4. S-F(R) - refers to regular stop-flow

SUMMARY OF EXPERIMENTS

Pig	S - F - Type	Substance
I	A - R B - R	Hydrochloric Acid
II	A - R B - R	Hydrochloric Acid
III	A - R B - R C - R	Al 1mg/kg bw
IV	A - R B - R C - R	Al 0.5mg/kg bw
V	A - R B - I C - I	Al 0.1mg/kg bw
VI	A - R B - R C - R	Al 0.1mg/kg bw
VII	A - R B - R C - R	Al 0.1mg/kg bw
VIII	A - R B - I C - R D - I	Al 0.1mg/kg bw

EXPERIMENTAL PLANS

EXPERIMENTAL PLAN

PIG: I

WEIGHT: 20 KG

TIME (Minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-60	9.15	1	
-50	9.25	2	
-40	9.35	3	
-30	9.45	4	
-25	9.50		S-F.I.A(R)
-15	10.00	5	
- 5	10.10	6	
0	10.15		HYDROCHLORIC ACID INJECTED
5	10.20	7	
15	10.30	8	
30	10.45		S-F.I.B(R)
35	10.50	9	
55	11.10	10	

EXPERIMENTAL PLAN

PIG: II

WEIGHT: 25 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-40	9.05	1	
-35	9.10	2	
-30	9.15		S-F.II.A(R)
-15	9.30	3	
-10	9.35	4	
0	9.45		HYDROCHLORIC ACID INJECTED
5	9.50	5	
15	10.00	6	
25	10.10		S-F.II.B(R)
40	10.25	7	
50	10.35	8	

EXPERIMENTAL PLAN

PIG: III

WEIGHT: 20 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-60	9.15	1	
	N/A	2	
-35	9.40	3	
	N/A	4	
	N/A		S-F.III.A(R)
0	10.15		ALUMINIUM INJECTED
15	10.30	5	
20	10.35	6	
	N/A		S-F.III.B(R)
45	11.00	7	
50	11.05	8	
	N/A		S-F.III.C(R)
75	11.30	9	

EXPERIMENTAL PLAN

PIG: IV

WEIGHT: 25 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-15	9.00	1	
-10	9.05	2	
0	9.15		ALUMINIUM INJECTED
10	9.25	3	
15	9.30	4	
25	9.40		S-F.IV.A(R)
35	9.50	5	
40	9.55	6	
50	10.05		S-F.IV.B(R)
60	10.15	7	
85	10.40	8	
90	10.45		S-F.IV.C(R)

EXPERIMENTAL PLAN

PIG: V

WEIGHT: 25 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-20	8.45	1	
-10	8.55	2	
0	9.05		ALUMINIUM INJECTED
5	9.10	3	
15	9.20	4	
20	9.25		S-F.V.A(R)
30	9.35	5	
35	9.40	6	
40	9.45		S-F.V.B(I)
55	10.00	7	
65	10.10		S-F.V.C(I)

EXPERIMENTAL PLAN

PIG: VI

WEIGHT: 40 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-40	9.35	1	
-30	9.45	2	
-25	9.50		S-F.VI.A(R)
-10	10.05	3	
0	10.15		ALUMINIUM INJECTED
5	10.20	4	
10	10.25	5	
20	10.35		S-F.VI.B(R)
35	10.50	6	
40	10.55	7	
55	11.10		S-F.VI.C(R)
60	11.15	8	

EXPERIMENTAL PLAN

PIG: VII

WEIGHT: 19 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-35	8.55	1	
-25	9.05	2	
-20	9.10		S-F.VII.A(R)
-10	9.20	3	
0	9.30		ALUMINIUM INJECTED
5	9.35	4	
10	9.40	5	
15	9.45		S-F.VII.B(R)
25	9.55	6	
30	10.00	7	
40	10.10		S-F.VII.C(R)
55	10.25	8	
65	10.35	9	

EXPERIMENTAL PLAN

PIG: VIII

WEIGHT: 34 KG

TIME (minutes)	TIME (a.m)	FREE-FLOW SAMPLE	STOP-FLOW SAMPLE
-10	9.30	1	
- 5	9.35	2	
0	9.40		ALUMINIUM INJECTED
5	9.45	3	
10	9.50	4	
20	10.00		S-F.VIII.A(R)
25	10.05	5	
30	10.10	6	
35	10.15		S-F.VIII.B(I)
50	10.30	7	
70	10.50		S-F.VIII.C(R)
80	11.00	8	
85	11.05	9	
90	11.10		S-F.VIII.D(I)
105	11.25	10	

SINGLE KIDNEY CLEARANCE

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG I

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	29	644	131
2	16	756	131
3	47	278	131
4	57	277	131
5	32	335	136
6	25	413	142
HYDROCHLORIC ACID INJECTED			
7	20	509	140
8	51	274	140
9	46	270	141
10	30	349	147

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG II

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	29	420	119
2	34	366	118
3	34	335	118
4	38	303	123
HYDROCHLORIC ACID INJECTED			
5	48	249	124
6	44	265	125
7	43	267	131
8	40	285	132

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG III

SAMPLE	URINE VOLUME (ml)	URINARY CREATITINE (µmol/L)	PLASMA CREATININE (µmol/L)
1	30	392	123
2	31	422	125
3	33	400	129
4	24	524	124
ALUMINIUM INJECTED			
5	44	319	129
6	23	504	140
7	31	508	136
8	41	382	130
9	N/A	434	131

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG IV

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	30	439	100
2	23	538	100
ALUMINIUM INJECTED			
3	30	501	100
4	26	496	101
5	23	673	103
6	20	755	104
7	44	345	106
8	23	694	105

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG V

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	47	382	122
2	31	472	123
ALUMINIUM INJECTED			
3	16	564	126
4	24	581	123
5	41	421	118
6	36	477	131
7	25	681	132

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG VI

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	22	1086	120
2	30	706	125
3	39	532	129
ALUMINIUM INJECTED			
4	45	512	126
5	42	506	130
6	46	480	128
7	44	468	131
8	58	404	127

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG VII

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	42	243	106
2	33	290	109
3	44	236	111
ALUMINIUM INJECTED			
4	29	344	108
5	28	314	110
6	25	346	112
7	22	444	114
8	35	286	113
9	37	256	117

SINGLE KIDNEY CREATININE CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG VIII

SAMPLE	URINE VOLUME (ml)	URINARY CREATININE (μmol/L)	PLASMA CREATININE (μmol/L)
1	40	390	132
2	47	432	133
ALUMINIUM INJECTED			
3	18	842	126
4	18	926	141
5	18	950	130
6	13	1200	130
7	15	1074	147
8	23	738	143
9	18	796	133
10	36	498	136

SINGLE KIDNEY INULIN CLEARANCE

ALL SAMPLES COLLECTED OVER 5 MINUTES

PIG VIII

SAMPLE	URINE VOLUME (ml)	URINARY INULIN (mg/dL)	PLASMA INULIN (mg/dL)
1	40	140	34
2	47	144	32
ALUMINIUM INJECTED			
3	18	252	26
4	18	251	25
5	18	224	24
6	13	263	22
7	15	238	19
8	23	133	19
9	18	170	19
10	36	117	24

STOP-FLOW DATA

PIG I

STOP-FLOW DATA - I.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.67	1.67
2	1.36	3.03
3	1.23	4.26
4	1.18	5.44
5	1.08	6.52
6	1.00	7.52
7	0.98	8.50
8	0.78	9.28
9	0.78	10.06
10	0.94	11.00
11	0.98	11.98
12	1.07	13.05
13	1.07	14.12
14	0.87	14.99
15	0.87	15.86
16	0.87	16.73
17	0.84	17.57
18	0.83	18.40
19	0.87	19.27
20	2.03	21.30

PIG I

STOP-FLOW DATA - I.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	52	0.78	371
2	34	0.78	504
3	22	0.69	511
4	13	0.54	483
5	12	0.41	511
6	20	0.36	455
7	N/A	0.30	476
8	N/A	0.41	441
9	N/A	0.31	434
10	54	0.28	399
11	64	0.22	364
12		0.22	343
13		0.19	350
14		0.17	350
15		0.17	357
16		0.16	336
17		0.19	392
18		0.20	350
19		0.20	336
20		0.28	294

PIG I

STOP-FLOW DATA - I.A

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.70
2	"	1.97
3	"	1.43
4	"	0.77
5	"	0.37
6	"	0.52
7	"	0.88
8	"	1.45
9	"	1.66
10	"	2.05
11	"	2.53
12	"	2.88
13	"	2.95
14	"	2.97
15	"	2.94
16	"	2.83
17	"	2.86
18	"	2.94
19	"	3.00
20	"	3.21

PIG I

STOP-FLOW DATA - I.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.05	1.05
2	1.19	2.24
3	1.16	3.40
4	0.83	4.23
5	0.99	5.22
6	0.85	6.07
7	0.72	6.79
8	0.95	7.74
9	0.81	8.55
10	1.11	9.66
11	0.85	10.51
12	0.93	11.44
13	0.74	12.18
14	1.03	13.21
15	0.86	14.07
16	0.57	14.64
17	0.62	15.26
18	0.74	16.00
19	0.56	16.56
20	1.57	18.13

PIG I

STOP-FLOW DATA - I.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	39	0.30	508
2	36	0.33	494
3	24	0.28	528
4	25	0.23	540
5	9	0.23	542
6	7	0.22	506
7	9	0.25	502
8	14	0.23	524
9	18	0.23	508
10	25	0.22	520
11		0.19	530
12		0.19	478
13		0.19	484
14		0.16	474
15		0.17	512
16		0.19	492
17		0.19	496
18		0.19	512
19		0.20	574
20		0.19	678

PIG I

STOP-FLOW DATA - I.B

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.20
2	"	1.97
3	"	1.51
4	"	1.08
5	"	0.64
6	"	0.41
7	"	0.43
8	"	0.63
9	"	0.87
10	"	1.42
11	"	1.82
12	"	2.24
13	"	2.29
14	"	2.31
15	"	2.30
16	"	2.30
17	"	2.33
18	"	2.36
19	"	2.31
20	"	2.37

PIG II

STOP-FLOW DATA - II.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.20	1.20
2	1.44	2.64
3	1.68	4.32
4	1.47	5.79
5	1.44	7.23
6	1.51	8.74
7	1.76	10.50
8	1.40	11.90
9	1.66	13.56
10	1.83	15.39
11	1.57	16.96
12	1.36	18.32
13	1.57	19.89
14	1.57	21.46
15	1.62	23.08
16	0.95	24.03
17	1.05	25.08
18	1.21	26.29
19	1.39	27.68
20	2.11	29.79

PIG II

STOP-FLOW DATA - II.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	62	1.01	354
2	58	1.09	356
3	35	1.30	536
4	21	1.15	528
5	13	1.09	486
6	17	0.72	464
7	36	0.54	438
8	55	0.50	440
9	69	0.40	412
10	64	0.34	370
11		0.31	362
12		0.25	394
13		0.44	388
14		0.58	342
15		0.75	320
16		0.92	304
17		1.13	284
18		1.58	276
19		1.46	310
20		1.44	260

PIG II

STOP-FLOW DATA - II.A

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.66
2	"	2.74
3	"	2.22
4	"	1.13
5	"	0.95
6	"	0.36
7	"	0.78
8	"	1.89
9	"	2.42
10	"	2.59
11	"	2.51
12	"	2.12
13	"	2.86
14	"	2.80
15	"	3.00
16	"	3.16
17	"	3.23
18	"	4.58
19	"	3.80
20	"	3.35

PIG II

STOP-FLOW DATA - II.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.92	1.92
2	2.61	4.53
3	1.63	6.16
4	1.72	7.88
5	1.73	9.61
6	1.61	11.22
7	1.82	13.04
8	1.72	14.76
9	1.70	16.46
10	2.00	18.46
11	1.13	19.59
12	1.49	21.08
13	1.59	22.67
14	1.79	24.46
15	1.27	25.73
16	1.15	26.88
17	1.08	27.96
18	1.28	29.24
19	1.37	30.61
20	2.69	33.30

PIG II

STOP-FLOW DATA - II.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	62	0.23	296
2	52	0.26	324
3	31	0.50	408
4	16	0.21	324
5	12	0.27	384
6	17	0.25	404
7	30	0.28	352
8	43	0.10	348
9	52	0.14	318
10	60	0.09	324
11		0.18	308
12		0.20	334
13		0.25	294
14		0.29	300
15		0.33	338
16		0.73	306
17		0.44	306
18		N/A	333
19		0.59	299
20		0.61	316

PIG II

STOP-FLOW DATA - II.B

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.92
2	"	2.90
3	"	3.84
4	"	1.39
5	"	0.70
6	"	0.48
7	"	0.81
8	"	1.83
9	"	2.65
10	"	3.27
11	"	3.07
12	"	3.57
13	"	2.97
14	"	2.90
15	"	2.90
16	"	N/A
17	"	2.93
18	"	N/A
19	"	3.38
20	"	3.49

PIG III

STOP-FLOW DATA - III.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.35	2.35
2	2.07	4.42
3	1.77	6.19
4	1.60	7.79
5	1.48	9.27
6	1.38	10.65
7	1.37	12.02
8	1.36	13.38
9	1.36	14.74
10	1.31	16.05
11	1.32	17.37
12	1.35	18.72
13	1.36	20.08
14	1.36	21.44
15	1.34	22.78
16	1.31	24.09
17	1.46	25.55
18	1.30	26.85
19	1.40	28.25
20	1.51	29.76

PIG III

STOP-FLOW DATA - III.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (µmol/L)
1	34	5.13	393
2	24	5.99	531
3	14	5.86	546
4	8	4.73	495
5	8	3.76	483
6	18	3.35	477
7	24	2.57	465
8	40	1.69	423
9	42	1.17	423
10	38	1.06	432
11		1.08	426
12		1.28	435
13		1.78	432
14		2.37	369
15		2.78	387
16		3.32	369
17		3.70	366
18		3.92	348
19		4.19	378
20		4.33	318

PIG III

STOP-FLOW DATA - III.A

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.12
2	"	1.78
3	"	0.98
4	"	0.41
5	"	0.21
6	"	0.71
7	"	1.63
8	"	2.56
9	"	2.73
10	"	2.46
11	"	2.35
12	"	2.48
13	"	2.74
14	"	2.56
15	"	2.74
16	"	2.76
17	"	2.62
18	"	2.51
19	"	2.57
20	"	2.54

PIG III

STOP-FLOW DATA - III.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.94	1.94
2	1.78	3.72
3	1.72	5.44
4	1.44	6.88
5	1.51	8.39
6	1.39	9.78
7	1.38	11.16
8	1.31	12.47
9	1.59	14.06
10	1.58	15.64
11	2.42	18.06
12	1.43	19.49
13	1.14	20.63
14	1.51	22.14
15	1.80	23.94
16	1.44	25.38
17	1.71	27.09
18	1.63	28.72
19	1.67	30.39
20	1.46	31.85

PIG III

STOP-FLOW DATA - III.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	35	1.87	405
2	29	1.89	483
3	17	1.90	507
4	8	1.57	507
5	5	1.25	465
6	12	0.96	447
7	21	0.75	462
8	31	0.64	420
9	39	0.44	378
10	40	0.50	387
11		0.49	354
12		0.73	309
13		0.85	291
14		1.06	312
15		1.29	312
16		1.55	N/A
17		1.76	N/A
18		1.72	303
19		1.81	309
20		1.87	333

PIG III

STOP-FLOW DATA - III.B

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	196	2.53
2	266	2.48
3	375	1.73
4	420	0.93
5	420	0.49
6	441	0.55
7	385	1.12
8	280	2.03
9	371	2.68
10	175	2.77
11	189	3.40
12	140	3.31
13	140	3.33
14	70	3.48
15	126	3.34
16	126	3.42
17	105	3.55
18	175	3.29
19	140	3.06
20	105	3.15

PIG III

STOP-FLOW DATA - III.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.48	2.48
2	2.15	4.63
3	1.71	6.34
4	1.40	7.74
5	1.37	9.11
6	1.55	10.66
7	1.51	12.17
8	1.83	14.00
9	1.79	15.79
10	2.29	18.08
11	2.33	20.41
12	2.26	22.67
13	1.76	24.43
14	1.78	26.21
15	1.64	27.85
16	1.74	29.59
17	1.53	31.12
18	2.61	33.73
19	2.84	36.57
20	2.83	39.40

PIG III

STOP-FLOW DATA - III.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	49	1.63	189
2	39	2.15	246
3	24	1.97	294
4	16	1.75	279
5	11	1.47	267
6	13	N/A	255
7	16	1.06	258
8	26	0.89	237
9	42	0.66	228
10	52	0.49	219
11		0.41	207
12		0.52	198
13		0.58	189
14		0.70	156
15		0.78	210
16		0.90	198
17		1.01	183
18		1.08	180
19		1.18	180
20		1.34	180

PIG III

STOP-FLOW DATA - III.C

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	175	3.23
2	70	3.24
3	140	2.20
4	196	1.46
5	301	0.98
6	280	N/A
7	266	0.53
8	231	1.31
9	231	2.05
10	126	2.67
11	161	2.92
12	126	3.08
13	91	2.99
14	91	3.19
15	91	3.29
16	70	3.46
17	105	3.50
18	105	3.57
19	91	3.50
20	91	3.60

PIG IV

STOP-FLOW DATA - IV.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.30	2.30
2	2.02	4.32
3	1.81	6.13
4	1.45	7.58
5	1.34	8.92
6	1.75	10.67
7	1.69	12.36
8	1.71	14.07
9	1.84	15.91
10	1.74	17.65
11	1.46	19.11
12	1.69	20.80
13	1.76	22.56
14	1.80	24.36
15	1.75	26.11
16	1.62	27.73
17	1.64	29.37
18	1.87	31.24
19	2.09	33.33
20	3.15	36.48

PIG IV

STOP-FLOW DATA - IV.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	46	3.30	546
2	32	3.79	730
3	16	3.44	686
4	10	3.02	644
5	12	2.70	642
6	20	2.40	624
7	38	2.04	556
8	50	1.76	528
9	52	1.71	502
10	52	1.69	496
11	52	1.70	462
12	56	1.77	440
13	56	1.91	414
14	58	2.14	394
15	54	2.33	370
16	60	2.51	352
17	62	2.77	342
18	64	3.09	342
19	56	3.34	340
20	62	3.77	326

PIG IV

STOP-FLOW DATA - IV.A

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	150	2.73
2	202	2.24
3	193	1.26
4	202	0.57
5	202	0.38
6	225	0.88
7	179	1.73
8	150	2.53
9	150	2.57
10	171	2.75
11	144	2.84
12	112	3.18
13	121	3.23
14	127	3.38
15	112	3.49
16	127	3.67
17	121	3.81
18	150	3.91
19	127	3.88
20	127	3.90

PIG IV

STOP-FLOW DATA - IV.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.25	2.25
2	1.90	4.15
3	1.60	5.75
4	1.28	7.03
5	1.24	8.27
6	1.05	9.32
7	0.87	10.19
8	1.24	11.43
9	1.79	13.22
10	2.04	15.26
11	1.31	16.57
12	1.32	17.89
13	1.29	19.18
14	1.37	20.55
15	2.02	22.57
16	1.98	24.55
17	1.97	26.52
18	2.11	28.63
19	1.91	30.54
20	2.85	33.39

PIG IV

STOP-FLOW DATA - IV.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	46	0.72	450
2	28	0.92	560
3	20	0.89	524
4	16	0.75	498
5	10	0.66	497
6	10	0.62	469
7	12	0.66	455
8	20	0.59	444
9	30	0.59	428
10	36	0.39	380
11		0.33	350
12		0.33	322
13		0.33	322
14		0.36	356
15		0.36	334
16		0.33	320
17		0.36	310
18		0.49	294
19		0.53	272
20		0.59	264

PIG IV

STOP-FLOW DATA - IV.B

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	105	3.46
2	105	2.77
3	112	2.10
4	99	1.09
5	112	0.64
6	112	0.49
7	112	0.52
8	112	0.87
9	105	1.36
10	99	2.30
11	81	2.80
12	90	2.80
13	90	3.07
14	74	3.00
15	90	3.06
16	81	2.87
17	81	3.12
18	81	3.46
19	81	3.64
20	81	3.75

PIG IV

STOP-FLOW DATA - IV.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.97	1.97
2	2.16	4.13
3	1.40	5.53
4	1.94	7.47
5	1.85	9.32
6	1.42	10.74
7	1.91	12.65
8	1.77	14.42
9	1.58	16.00
10	1.56	17.56
11	1.66	19.22
12	1.55	20.77
13	2.03	22.80
14	1.95	24.75
15	1.77	26.52
16	1.42	27.94
17	1.44	29.38
18	1.45	30.83
19	1.93	32.76
20	2.88	35.64

PIG IV

STOP-FLOW DATA - IV.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	37	0.36	534
2	32	0.36	636
3	20	0.33	604
4	11	0.33	560
5	5	0.33	540
6	7	0.33	516
7	15	0.33	486
8	28	0.30	452
9	39	0.30	430
10	45	0.25	400
11		0.21	404
12		0.25	384
13		0.23	380
14		0.23	346
15		0.25	322
16		0.25	320
17		0.30	316
18		0.34	316
19		0.44	318
20		0.52	328

PIG IV

STOP-FLOW DATA - IV.C

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	220	2.88
2	335	2.34
3	287	1.73
4	270	0.98
5	235	0.45
6	235	0.35
7	270	0.96
8	220	1.86
9	185	2.36
10	137	2.85
11	137	2.73
12	120	3.08
13	137	3.13
14	103	3.39
15	68	3.47
16	85	3.75
17	120	3.78
18	170	4.03
19	137	4.10
20	103	4.03

PIG V

STOP-FLOW DATA - V.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.21	2.21
2	1.74	3.95
3	1.54	5.49
4	1.32	6.81
5	1.41	8.22
6	1.41	9.63
7	1.57	11.20
8	1.50	12.70
9	1.66	14.36
10	1.47	15.83
11	1.60	17.43
12	1.64	19.07
13	1.51	20.58
14	1.50	22.08
15	1.46	23.54
16	1.59	25.13
17	1.48	26.61
18	1.44	28.05
19	1.55	29.60
20	2.52	32.12

PIG V

STOP-FLOW DATA - V.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	56	5.12	700
2	52	5.61	628
3	34	5.78	696
4	22	5.49	672
5	16	4.75	664
6	16	4.16	665
7	26	3.53	644
8	36	2.85	604
9	50	2.05	556
10	62	1.65	544
11	56	1.42	504
12	54	1.37	520
13	54	1.45	516
14	54	1.79	488
15	56	1.96	484
16	54	2.19	456
17	54	2.36	456
18	60	2.56	420
19	62	2.85	416
20	58	2.99	428

PIG V

STOP-FLOW DATA - V.A

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	43	3.83
2	70	3.16
3	92	2.29
4	100	1.53
5	88	0.84
6	72	0.59
7	70	0.91
8	54	1.98
9	48	2.79
10	13	3.35
11	11	3.33
12	15	3.22
13	17	3.03
14	22	3.10
15	20	3.11
16	8	3.11
17	8	3.22
18	8	3.24
19	13	3.43
20	13	3.15

PIG V

STOP-FLOW DATA - V.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.36	2.36
2	2.09	4.45
3	1.80	6.25
4	1.51	7.76
5	1.53	9.29
6	1.77	11.06
7	1.84	12.90
8	1.76	14.66
9	1.70	16.36
10	1.68	18.04
11	1.38	19.42
12	1.49	20.91
13	1.55	22.46
14	1.44	23.90
15	1.54	25.44
16	1.53	26.97
17	1.61	28.58
18	1.61	30.19
19	1.59	31.78
20	2.35	34.13

PIG V

STOP-FLOW DATA - V.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	24	1.62	668
2	26	1.33	768
3	20	1.22	840
4	14	1.14	856
5	10	1.08	882
6	10	1.00	800
7	16	0.94	808
8	24	0.88	736
9	36	0.68	684
10	42	0.68	640
11		0.57	624
12		0.68	624
13		0.66	628
14		0.77	624
15		0.85	616
16		0.94	580
17		1.11	564
18		1.34	544
19		1.48	524
20		1.79	536

PIG V

STOP-FLOW DATA - V.B

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	70	1.41
2	70	2.03
3	70	1.67
4	74	1.13
5	74	0.51
6	74	0.34
7	61	0.56
8	43	1.40
9	52	2.47
10	22	3.04
11	24	3.28
12	17	2.98
13	24	3.04
14	22	2.90
15	22	2.81
16	17	3.00
17	22	3.06
18	26	3.04
19	30	3.09
20	39	3.04

PIG V

STOP-FLOW DATA - V.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.16	2.16
2	2.03	4.19
3	1.67	5.86
4	1.57	7.43
5	2.23	9.66
6	1.55	11.21
7	1.63	12.84
8	1.54	14.38
9	1.58	15.96
10	1.75	17.71
11	1.46	19.17
12	1.17	20.34
13	1.08	21.42
14	1.31	22.73
15	1.10	23.83
16	1.34	25.17
17	1.02	26.19
18	1.06	27.25
19	1.39	28.64
20	2.02	30.66

PIG V

STOP-FLOW DATA - V.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	22	1.14	786
2	28	0.94	864
3	22	0.91	892
4	14	0.91	866
5	10	0.91	846
6	10	0.85	902
7	14	0.80	894
8	22	0.91	812
9	30	0.68	848
10	38	0.54	764
11		0.46	772
12		0.48	860
13		0.54	864
14		0.63	860
15		0.71	896
16		0.88	910
17		1.05	868
18		1.22	780
19		1.48	824
20		1.71	858

PIG V

STOP-FLOW DATA - V.C

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	76	1.26
2	61	2.61
3	43	2.20
4	65	1.55
5	65	0.76
6	63	0.54
7	61	0.67
8	43	1.60
9	39	2.39
10	43	2.93
11	43	3.23
12	46	2.23
13	35	2.99
14	52	2.93
15	52	2.92
16	52	2.70
17	52	2.71
18	54	2.59
19	57	2.61
20	52	2.59

PIG VI

STOP-FLOW DATA - VI.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.10	2.10
2	1.89	3.99
3	1.97	5.96
4	1.94	7.90
5	1.85	9.75
6	1.69	11.44
7	1.68	13.12
8	1.65	14.77
9	1.65	16.42
10	2.01	18.43
11	1.98	20.41
12	1.55	21.96
13	1.02	22.98
14	1.98	24.96
15	1.48	26.44
16	2.24	28.68
17	1.42	30.10
18	2.14	32.24
19	1.45	33.69
20	2.78	36.47

PIG VI

STOP-FLOW DATA - VI.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (µmol/L)
1	42	2.87	726
2	34	3.09	920
3	24	3.39	1040
4	13	2.97	936
5	11	2.57	968
6	19	2.19	932
7	28	1.81	802
8	N/A	1.55	N/A
9	51	1.29	758
10	55	1.14	720
11		1.19	768
12		0.98	700
13		0.94	758
14		1.20	626
15		1.02	612
16		1.10	556
17		1.35	512
18		1.35	474
19		1.55	N/A
20		1.74	436

PIG VI

STOP-FLOW DATA - VI.A

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	N/D	2.39
2	"	2.06
3	"	1.38
4	"	0.78
5	"	0.57
6	"	0.50
7	"	1.07
8	"	2.22
9	"	2.71
10	"	3.03
11	"	3.03
12	"	3.16
13	"	3.34
14	"	3.34
15	"	3.39
16	"	3.60
17	"	3.71
18	"	3.79
19	"	3.77
20	"	3.77

PIG VI

STOP-FLOW DATA - VI.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.82	1.82
2	1.86	3.68
3	1.75	5.43
4	1.62	7.05
5	1.97	9.02
6	1.63	10.65
7	1.36	12.01
8	1.47	13.48
9	1.42	14.90
10	1.74	16.64
11	1.50	18.14
12	1.63	19.77
13	1.60	21.37
14	1.81	23.18
15	1.62	24.80
16	1.60	26.40
17	1.62	28.02
18	1.73	29.75
19	2.14	31.89
20	2.91	34.80

PIG VI

STOP-FLOW DATA - VI.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	42	0.80	536
2	39	0.68	598
3	31	0.82	680
4	23	0.80	706
5	13	0.70	720
6	10	0.62	716
7	13	0.65	716
8	18	0.56	716
9	31	0.56	710
10	36	0.51	690
11		0.40	636
12		0.40	626
13		0.46	644
14		0.37	646
15		0.40	630
16		0.36	644
17		0.43	622
18		0.37	596
19		0.43	578
20		0.55	602

PIG VI

STOP-FLOW DATA - VI.B

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	140	2.98
2	225	3.04
3	250	2.55
4	250	2.11
5	280	1.23
6	280	0.72
7	250	0.68
8	305	0.94
9	250	1.80
10	225	2.43
11	225	2.92
12	165	3.08
13	165	3.16
14	165	3.20
15	165	3.21
16	165	3.25
17	165	3.37
18	165	3.42
19	165	3.49
20	165	3.45

PIG VI

STOP-FLOW DATA - VI.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.16	2.16
2	2.28	4.44
3	2.08	6.52
4	1.97	8.49
5	1.85	10.34
6	2.05	12.39
7	2.19	14.58
8	1.93	16.51
9	1.99	18.50
10	2.25	20.75
11	1.99	22.74
12	1.92	24.66
13	1.91	26.57
14	1.74	28.31
15	1.72	30.03
16	1.99	32.02
17	1.97	33.99
18	2.01	36.00
19	1.95	37.95
20	2.72	40.67

PIG VI

STOP-FLOW DATA - VI.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	43	0.57	520
2	37	0.64	568
3	24	0.68	632
4	17	0.58	620
5	12	0.56	628
6	13	0.49	642
7	22	0.49	616
8	33	0.55	568
9	42	0.37	546
10	47	0.33	522
11		0.34	508
12		0.37	518
13		0.32	508
14		0.32	498
15		0.33	476
16		0.34	472
17		0.39	452
18		0.58	444
19		0.48	436
20		0.48	438

PIG VI

STOP-FLOW DATA - VI.C

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	28	3.01
2	64	2.77
3	70	1.97
4	104	1.44
5	100	0.91
6	72	0.74
7	106	1.12
8	80	1.98
9	58	2.53
10	72	2.99
11	56	3.12
12	68	3.11
13	54	3.18
14	56	3.32
15	56	3.32
16	54	3.41
17	N/A	3.52
18	60	3.50
19	64	3.57
20	50	3.70

PIG VII

STOP-FLOW DATA - VII.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.46	1.46
2	1.45	2.91
3	1.73	4.64
4	1.71	6.35
5	1.96	8.31
6	1.47	9.78
7	1.55	11.33
8	1.72	13.05
9	1.59	14.64
10	1.63	16.27
11	1.19	17.46
12	1.55	19.01
13	1.62	20.63
14	1.68	22.31
15	1.65	23.96
16	1.53	25.49
17	1.49	26.98
18	1.69	28.67
19	1.63	30.30
20	3.10	33.40

PIG VII

STOP-FLOW DATA - VII.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	50	4.04	266
2	46	3.90	273
3	37	4.37	336
4	22	5.12	392
5	12	4.53	343
6	13	3.90	350
7	20	3.38	336
8	30	2.86	301
9	46	2.21	294
10	51	1.70	254
11		1.48	266
12		1.27	266
13		1.23	266
14		1.20	252
15		1.30	238
16		1.33	224
17		1.51	210
18		1.68	189
19		1.95	210
20		2.38	182

PIG VII

STOP-FLOW DATA - VII.A

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	N/D	3.19
2	"	3.17
3	"	3.19
4	"	2.45
5	"	1.32
6	"	0.78
7	"	0.79
8	"	1.25
9	"	2.20
10	"	2.77
11	"	3.08
12	"	3.16
13	"	3.20
14	"	3.26
15	"	3.32
16	"	3.36
17	"	3.41
18	"	3.52
19	"	3.61
20	"	3.66

PIG VII

STOP-FLOW DATA - VII.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.09	1.09
2	1.45	2.54
3	1.60	4.14
4	1.74	5.88
5	1.52	7.40
6	1.49	8.89
7	1.48	10.37
8	1.78	12.15
9	1.22	13.37
10	1.68	15.05
11	1.62	16.67
12	1.39	18.06
13	1.62	19.68
14	1.40	21.08
15	1.53	22.61
16	1.38	23.99
17	1.34	25.33
18	1.44	26.77
19	1.42	28.19
20	2.57	30.76

PIG VII

STOP-FLOW DATA - VII.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	42	1.03	324
2	39	1.00	327
3	34	1.06	381
4	20	1.12	417
5	14	0.95	414
6	12	0.85	402
7	15	0.76	402
8	24	0.70	378
9	36	0.64	354
10	46	0.52	330
11		0.45	327
12		0.39	318
13		0.44	303
14		0.42	309
15		0.45	270
16		0.50	234
17		0.56	270
18		0.56	276
19		0.70	249
20		0.88	240

PIG VII

STOP-FLOW DATA - VII.B

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	43	2.82
2	53	2.96
3	69	2.90
4	75	2.50
5	75	1.43
6	75	0.92
7	73	0.69
8	66	0.95
9	60	1.74
10	55	2.40
11	46	2.95
12	50	3.09
13	46	3.17
14	43	3.29
15	43	3.36
16	43	3.48
17	41	3.66
18	41	3.65
19	39	3.74
20	39	3.75

PIG VII

STOP-FLOW DATA - VII.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.71	1.71
2	1.57	3.28
3	1.54	4.82
4	1.16	5.98
5	1.45	7.43
6	1.54	8.97
7	1.36	10.33
8	1.58	11.91
9	1.67	13.58
10	1.78	15.36
11	1.53	16.89
12	1.43	18.32
13	1.24	19.56
14	1.16	20.72
15	1.09	21.81
16	1.43	23.24
17	1.30	24.54
18	1.45	25.99
19	1.24	27.23
20	2.31	29.54

PIG VII

STOP-FLOW DATA - VII.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	37	0.28	381
2	33	0.40	408
3	25	0.38	447
4	17	0.36	468
5	9	0.29	468
6	10	0.25	441
7	15	0.26	426
8	25	0.20	417
9	37	0.20	393
10	44	0.16	357
11		0.15	360
12		0.12	339
13		0.17	333
14		0.13	315
15		0.16	327
16		0.21	294
17		0.21	294
18		0.23	279
19		0.30	288
20		0.35	282

PIG VII

STOP-FLOW DATA - VII.C

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	52	3.14
2	55	3.35
3	57	2.97
4	64	3.40
5	66	1.69
6	64	0.95
7	62	0.85
8	62	1.25
9	53	2.18
10	41	2.80
11	41	3.29
12	39	3.52
13	39	3.52
14	37	3.74
15	34	3.77
16	32	3.83
17	34	3.98
18	32	3.96
19	32	3.96
20	30	4.06

PIG VIII

STOP-FLOW DATA - VIII.A

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.88	1.88
2	1.94	3.82
3	1.81	5.63
4	1.62	7.25
5	1.66	8.91
6	1.74	10.65
7	1.59	12.24
8	1.60	13.84
9	1.60	15.44
10	1.40	16.84
11	1.52	18.36
12	1.35	19.71
13	1.43	21.14
14	1.67	22.81
15	1.31	24.12
16	1.36	25.48
17	1.26	26.74
18	1.30	28.04
19	1.47	29.51
20	2.38	31.89

PIG VIII

STOP-FLOW DATA - VIII.A

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	35	0.47	1154
2	20	0.42	1004
3	11	0.26	964
4	11	0.26	990
5	12	0.30	944
6	15	0.31	938
7	27	0.27	900
8	40	0.23	808
9	55	0.25	686
10	67	0.23	642
11		0.22	630
12		0.18	620
13		0.37	518
14		0.20	558
15		0.29	480
16		0.21	558
17		0.25	510
18		0.34	480
19		0.29	548
20		0.54	490

PIG VIII

STOP-FLOW DATA - VIII.A

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	316	1.71
2	433	1.22
3	399	0.65
4	368	0.62
5	333	0.55
6	316	0.47
7	268	0.74
8	234	1.76
9	169	2.79
10	152	3.51
11	152	3.72
12	152	3.72
13	100	3.58
14	134	3.60
15	117	3.67
16	117	3.69
17	117	3.76
18	117	3.83
19	100	3.84
20	82	3.75

PIG VIII

STOP-FLOW DATA - VIII.B

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	2.10	2.10
2	1.78	3.88
3	1.78	5.66
4	1.69	7.35
5	1.51	8.86
6	1.45	10.31
7	1.63	11.94
8	1.79	13.73
9	1.60	15.33
10	1.83	17.16
11	1.85	19.01
12	1.82	20.83
13	1.78	22.61
14	1.34	23.95
15	1.43	25.38
16	1.53	26.91
17	1.62	28.53
18	2.00	30.53
19	1.51	32.04
20	1.70	33.74

PIG VIII

STOP-FLOW DATA - VIII.B

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	40	0.19	940
2	41	0.25	1124
3	28	0.23	1110
4	21	0.33	1074
5	14	0.23	956
6	14	0.20	1130
7	20	0.21	1116
8	30	0.19	886
9	45	0.20	926
10	54	0.17	886
11		N/A	884
12		0.16	934
13		0.15	804
14		0.21	824
15		0.22	778
16		0.09	754
17		0.12	572
18		0.25	564
19		0.51	576
20		0.34	526

PIG VIII

STOP-FLOW DATA - VIII.B

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	375	2.09
2	346	2.61
3	384	1.86
4	355	1.31
5	365	0.89
6	365	0.67
7	336	0.72
8	269	1.34
9	240	2.41
10	163	3.01
11	144	3.56
12	153	3.83
13	163	3.56
14	153	3.81
15	144	3.69
16	144	3.84
17	163	4.09
18	144	4.25
19	144	4.18
20	182	3.95

PIG VIII

STOP-FLOW DATA - VIII.C

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.75	1.75
2	1.45	3.20
3	1.50	4.70
4	1.67	6.37
5	1.55	7.92
6	1.40	9.32
7	1.48	10.80
8	1.52	12.32
9	1.61	13.93
10	2.14	16.07
11	1.53	17.60
12	1.67	19.27
13	1.70	20.97
14	1.44	22.41
15	1.59	24.00
16	1.64	25.64
17	1.32	26.96
18	1.45	28.41
19	1.76	30.17
20	2.85	33.02

PIG VIII

STOP-FLOW DATA - VIII.C

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	35	0.17	896
2	26	0.26	1026
3	20	0.26	1010
4	12	0.20	1072
5	9	0.11	928
6	10	0.10	856
7	16	0.10	844
8	28	0.10	810
9	39	0.11	654
10	59	0.12	650
11		0.11	616
12		0.10	640
13		0.11	590
14		0.13	570
15		0.11	568
16		0.13	530
17		0.13	534
18		0.13	504
19		0.06	464
20		0.21	516

PIG VIII

STOP-FLOW DATA - VIII.C

SAMPLE	ALUMINIUM (μg/L)	CALCIUM (mg/dL)
1	192	1.81
2	240	1.46
3	279	0.92
4	288	0.58
5	250	0.50
6	250	0.35
7	240	0.42
8	192	0.94
9	173	2.08
10	125	3.03
11	125	3.33
12	106	3.62
13	96	3.31
14	115	3.51
15	106	3.35
16	115	3.52
17	106	3.45
18	106	3.68
19	96	3.67
20	106	3.64

PIG VIII

STOP-FLOW DATA - VIII.D

SAMPLE	URINE VOLUME (ml)	CUMULATIVE URINE VOLUME (ml)
1	1.64	1.64
2	2.17	3.81
3	2.11	5.92
4	2.20	8.12
5	2.07	10.19
6	1.95	12.14
7	1.97	14.11
8	2.04	16.15
9	2.08	18.23
10	2.50	20.73
11	1.70	22.43
12	1.88	24.31
13	1.99	26.30
14	1.96	28.26
15	1.69	29.95
16	1.83	31.78
17	1.75	33.53
18	1.77	35.30
19	1.64	36.94
20	2.52	39.46

PIG VIII

STOP-FLOW DATA - VIII.D

SAMPLE	SODIUM (mmol/L)	PHOSPHATE (mg/dL)	CREATININE (μmol/L)
1	33	0.14	798
2	38	0.20	858
3	28	0.20	712
4	18	0.19	856
5	12	0.15	796
6	13	0.16	818
7	24	0.19	766
8	36	0.13	760
9	53	0.10	610
10	66	0.09	668
11		0.09	586
12		0.05	632
13		0.09	566
14		0.08	550
15		0.13	538
16		0.12	526
17		0.16	474
18		0.20	446
19		0.19	472
20		0.28	474

PIG VIII

STOP-FLOW DATA - VIII.D

SAMPLE	ALUMINIUM (µg/L)	CALCIUM (mg/dL)
1	209	1.53
2	209	1.95
3	209	1.54
4	198	0.92
5	198	0.61
6	186	0.32
7	166	1.40
8	166	2.47
9	146	3.13
10	127	3.55
11	114	3.19
12	95	3.44
13	95	3.49
14	95	3.48
15	103	3.42
16	103	3.69
17	83	3.86
18	83	3.84
19	83	3.84
20	83	3.78

FRACTIONAL EXCRETION OF FILTERED CREATININE

PIG VIII

STOP-FLOW VIII.A

SAMPLE NO	CREATININE (μmol/L)	INULIN (mg/dL)	CR/INULIN URINE RATIO	INULIN/CR PLASMA RATIO	FR.EXC.FIL. CR %
1	1154	376	3.07	0.176	54.03
2	1004	345	2.91	"	51.23
3	964	279	3.46	"	60.83
4	990	251	3.94	"	69.44
5	994	229	4.12	"	72.58
6	938	216	4.34	"	76.45
7	900	207	4.35	"	76.55
8	808	184	4.39	"	77.31
9	686	171	4.01	"	70.63
10	642	163	3.94	"	69.34
11	630	140	4.50	"	79.23
12	620	137	4.53	"	79.68
13	518	139	3.73	"	65.61
14	558	146	3.82	"	67.29
15	480	125	3.84	"	67.61
16	558	127	4.39	"	77.35
17	510	126	4.05	"	71.26
18	480	114	4.21	"	74.13
19	548	117	4.68	"	82.46
20	490	132	3.71	"	65.35

PIG VIII

STOP-FLOW VIII.B

SAMPLE NO	CREATININE (µmol/L)	INULIN (mg/dL)	CR/INULIN URINE RATIO	INULIN/CR PLASMA RATIO	FR.EXC.FIL. CR %
1	940	211	4.45	0.169	75.39
2	1124	247	4.55	"	77.01
3	1110	210	5.29	"	89.45
4	1074	211	5.09	"	86.14
5	956	202	4.73	"	80.09
6	1130	200	5.65	"	95.62
7	1116	187	5.97	"	101.00
8	886	199	4.45	"	75.35
9	926	167	5.54	"	93.84
10	886	168	5.27	"	89.25
11	884	159	5.56	"	94.09
12	934	153	6.10	"	103.31
13	804	146	5.51	"	93.19
14	824	141	5.84	"	98.90
15	778	128	6.08	"	102.86
16	754	128	5.89	"	99.69
17	572	117	4.89	"	82.74
18	564	114	4.95	"	83.72
19	576	117	4.92	"	83.31
20	526	122	4.31	"	72.96

PIG VIII

STOP-FLOW VIII.C

SAMPLE NO	CREATININE (µmol/L)	INULIN (mg/dL)	CR/INULIN URINE RATIO	INULIN/CR PLASMA RATIO	FR.EXC.FIL. CR %
1	896	181	4.95	0.129	63.98
2	1026	206	4.98	"	64.37
3	1010	199	5.08	"	65.60
4	1072	180	5.96	"	76.98
5	928	165	5.62	"	72.69
6	856	154	5.56	"	71.84
7	844	152	5.55	"	71.77
8	810	141	5.74	"	74.25
9	654	132	4.95	"	64.04
10	650	121	5.37	"	69.43
11	616	116	5.31	"	68.64
12	640	110	5.82	"	75.20
13	590	113	5.22	"	67.49
14	570	100	5.70	"	73.67
15	568	99	5.74	"	74.16
16	530	90	5.89	"	76.11
17	534	88	6.07	"	78.43
18	504	84	6.00	"	77.55
19	464	83	5.59	"	72.26
20	516	86	6.00	"	77.55

PIG VIII

STOP-FLOW VIII.D

SAMPLE NO	CREATININE (μmol/L)	INULIN (mg/dL)	CR/INULIN URINE RATIO	INULIN/CR PLASMA RATIO	FR.EXC.FIL. CR %
1	798	161	4.96	0.143	70.91
2	858	175	4.90	"	70.04
3	712	165	4.32	"	61.65
4	856	152	5.63	"	80.45
5	796	149	5.34	"	76.32
6	818	148	5.53	"	78.96
7	766	145	5.28	"	75.47
8	760	136	5.59	"	79.83
9	610	134	4.55	"	65.03
10	668	113	5.91	"	84.45
11	586	120	4.88	"	69.76
12	632	128	4.94	"	70.54
13	566	120	4.72	"	67.38
14	550	110	5.00	"	71.43
15	538	99	5.43	"	77.63
16	526	102	5.16	"	73.67
17	474	100	4.74	"	67.71
18	446	91	4.90	"	70.02
19	472	90	5.24	"	74.92
20	474	92	5.15	"	73.60

COMPOSITION OF M199 MEDIUM (CUSTOMISED TO BE PHOSPHATE FREE) IN MG/L

Supplied by Highveld Biologicals, Johannesburg (Catalog No P06-cm)

NaCl	6800
KC	400
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2$	200
D-Glucose	1000
Phenol red	17
$NaHCO_3$	2200
DL-Alanine	50
L-Arginine HCl	70
DL-Aspartic acid	60
L-Cysteine HCl	0.1
L-Cystine	20
L-Glutamine	100
DL Glutamic acid H_2O	150
Glycine	50
L-Histidine HCl	20
L-Hydroxyproline	10
DL-Isoleucine	40
DL-Leucine	120
L-Lysine HCl	70
DL-Methionine	30
DL-Phenylalanine	50
L-Proline	40
DL-Serine	50
DL-Threonine	60
DL-Tryptophan	20
L-Tyrosine	40
DL-Valine	50
Glutathione	0.5
Sodium Acetate	50
$Fe(NO_3)_3$	0.1
Tween 80	20
Adenine sulphate	10
Guanine HCl	0.3
Hypoxanthine	0.3
Thymine	0.3
Uracil	0.3
Xanthine	0.3
ATP, disodium salt	1
AMP	0.2
Ascorbic acid	0.05
Biotin	0.01
Calciferol	0.1
D-Ca-Pantothenate	0.01
Choline chloride	0.5
Folic acid	0.01
i-Inositol	0.05
Menadione	0.01
Nicotinic acid	0.025
Nicotinamide	0.025
p-Aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamine HCl	0.01
DL-α-Tocopherol phosphate Na_2	0.01
Vitamin A	0.1
Cholesterol	0.2
2-Deoxyribose	0.5
D-Ribose	0.5

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