THE EFFECTS OF SOME LIGANDS ON ASPECTS OF CALCIUM AND MAGNESIUM METABOLISM

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Dedication

To my wife Marian, and baby Roderick.

Declaration

In accordance with regulation 2.4.15 I declare that the work reported in this thesis is my own, and that the thesis has been composed by myself.

D.A.H. Farningham

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ABSTRACT

In biological solutions, such as plasma, a small proportion of the calcium and magnesium present exists as free ions in equilibrium with a larger inactive fraction which is bound to protein or small diffusible ligands. The effects of ligands on the distribution of calcium and magnesium between these physico-chemical states, and their effects on the kinetics of these minerals in intact sheep and horses form the major part of this study. Trisodium ethylene diamine tetraacetate (EDTA), calcium and sodium borogluconate, and disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), all exogenous ligands of pharmacological and therapeutic interest were studied.

Estimation of total calcium and magnesium concentrations presents few problems. However extensive investigations of possible methods of estimating diffusible and ionic calcium and magnesium were necessary, as was the validation of a method of estimating glomerular filtration rate (GFR) in conscious intact sheep.

EDTA, largely in confirmation of published studies, caused an almost stoichiometric shift of plasma calcium into the non-ionized diffusible fraction, and resulted in stoichiometric increments in urinary calcium and EDTA. The renal clearance of EDTA was similar to GFR.

In vitro, calcium-borogluconate complex formation was demonstrated and synergism observed between borate and gluconate. During calcium borogluconate infusion a small increase in the concentration of ionized plasma calcium was accompanied by a larger increase in the non-ionized diffusible fraction. Administration of sodium borogluconate resulted in decreased total plasma calcium concentration, probably as a result of decreased renal tubular reabsorption of calcium complexes. Calcium infusion was also associated with reduced GFR, acidosis and slight hypomagnesaemia, the implications of which were discussed.

EHDP was found to form large non-diffusible calcium and magnesium complexes in vitro. This was due to aggregation of complexes and not protein binding. Infusion of EHDP resulted in increases in non-diffusible ultrafiltrable calcium in plasma and urine. Evidence also indicated that the renal clearance of EHDP was lower than GFR, presumably due to the low renal clearance of large molecules. Additionally, chronic administration of low doses of EHDP to horses caused a calciuresis too great to be attributable to simple filtration of complexes, suggesting a second mode of action. Limited evidence indicated a similar phenomenon in sheep.

Since ammonium chloride induced acidosis results in depletion of bone mineral and EHDP reduces calcium turnover in bone, these drugs were studied simultaneously in mice. Both substances caused significant loss of bone potassium (per gm dry weight); acidosis also reduced bone sodium and total bone fat. Since EHDP did not alter the effects of acidosis it seemed unlikely to be of clinical benefit. The potential of other diphosphonates was discussed.

CHAPTER I

INTRODUCTION

Calcium and magnesium are cations of particular interest in veterinary medicine. Dryerre and Greig (1925) suggested that cows suffering from milk fever had parathyroid deficiency. They went on to demonstrate an association between milk fever and hypocalcaemia (Dryerre and Greig 1928). Subsequently the presence of hypomagnesaemia in calves with milk tetany was demonstrated by Duncan, Huffman and Robinson (1935). Since these early studies further work has confirmed the original observations and provided a greater insight into calcium and magnesium homeostasis.

I. Calcium and magnesium fractions in blood and plasma

It has long been recognised that plasma calcium and magnesium exist as diffusible and non-diffusible fractions (Rona and Takahashi, 1911; Tschimber and Tschimber, 1924). The presence of a non-diffusible fraction was attributed to protein binding.

McLean and Hastings (1934a, 1934b) showed that calcium complexes are formed by proteins and some diffusible anions normally present in plasma. Plasma calcium and magnesium exists in three forms; protein bound, complexed and ionized. In man, of the total plasma calcium 54% is ultrafiltrable and 48% ionized (Walser, 1961a). Plasma magnesium is 69% ultrafiltrable and 55% ionized (Walser,

1961a).

Ionized calcium and magnesium

The biological significance of ionized calcium was not clearly demonstrated until McLean and Hastings (1934a) produced a reliable and accurate method of measuring the ionized component of plasma calcium. They showed (McLean and Hastings, 1934b) that substances which bind calcium reduce the contractility of the frog heart preparation. The clear inference was that the inotropic response of the myocardium was related to the calcium ion concentration. Other workers have shown that perfusion of isolated parathyroid gland *in vitro* and *in vivo* with blood containing ethylene diamine tetra-acetic acid (EDTA) caused a rapid increase in parathyroid hormone secretion, evidence that ionized and not EDTA bound calcium is the important mediator of PTH secretion (Care, Sherwood, Potts, Mayer and Aurbach, 1966; Sherwood, Potts, Care, Mayer and Aurbach, 1966). The important implication of these studies was that the biologically active component of plasma calcium was the ionized fraction. It seems likely that ionized magnesium is also the biologically active form. Direct evidence, of the type cited for calcium, is difficult to produce since ionized magnesium is more difficult to measure and substances which complex magnesium without also complexing calcium are not yet available.

The biological or chemical response to an ion is related to its activity in solution. The ionic activity divided by the concentration of an ion is its activity coefficient. The activity coefficient is also related to the radius of the hydrated ion and the ionic strength of the solution (Kielland, 1937). Since in

biological solutions ionic strength varies little, the activity coefficient is fairly constant. Ionic activity is therefore approximately proportional to ionized concentration. The activity coefficient for calcium is about 0.36, for magnesium it is a little greater as the hydrated magnesium ion is slightly larger (0.40; Neuman and Neuman, 1958). Since methods of determining ionized calcium and magnesium respond to the activity of the ions rather than their concentrations, ionic strength will influence such determinations. Usually this problem is overcome by measuring very dilute calcium or magnesium standards and samples in which concentration approximately equals activity; or by measuring standards and samples of similar ionic strength, thus obtaining an estimate of concentration.

Measurement of ionized calcium

In the past many methods have been used for measuring ionized calcium. The frog heart method (McLean and Hastings, 1934a) was the first and for many years the best method of measuring ionized calcium. Other bioassays have also been used including a rachitic cartilage calcification method (Yendt, Connor and Howard, 1955) and the blood clotting method of Soulier and Crosnier (1958).

Aequorin is a jelly fish protein which fluoresces in the presence of calcium ions. It has been used to measure ionized calcium in plasma (Izutsu and Felton, 1972). Unfortunately the method seems to be unsuitable for use in plasma as the amount of aequorin required binds sufficient calcium to cause large disturbances of the normal calcium binding equilibria in plasma. The method seems to be more suitable for estimating calcium ion

concentration in pure solutions containing strong calcium chelating agents such as EDTA and EGTA (ethyleneglycol bis-(2 aminoethyl-ether)tetra-acetic acid) (Izutsu, Felton, Siegel, Nicholls, Crawford, McGough and Yoda, 1974).

Several workers have successfully used spectrophotometric methods. The methods involve the formation of coloured calcium complexes by dyes such as tetra methyl murexide. When the dye is present in low concentrations, ionized calcium concentration is related to the amount of coloured complex formed (Rose, 1957; Walser, 1960; Pedersen 1970). The absorbancy change for changes in ionized calcium is small, so it is difficult to obtain reliable results. These methods require prior ultrafiltration of plasma and so introduce two stages and subsequent compounding of errors.

The first attempt to measure ionized calcium using an ion selective electrode was that of Neuhausen and Marshall (1922) who made a calcium-mercury amalgam electrode. However it was not specific enough for use in plasma. Commercial ion selective electrodes are now available which can quickly, and with reasonable accuracy, measure ionized calcium. Early versions suffered from interference by sodium and were poisoned by contact with protein. More recently these problems have been largely overcome. Flow through electrodes are now available which are microproccessor controlled and thermostatted, for example the Nova-2 (American Hospital Supply UK Ltd) and SS-20 (Orion Research) which offer simple methods of ionized calcium estimation. Several types of electrode have been produced with liquid (Ross, 1967), solid state (Schwartz, 1975) and PVC membrane (Pors Nielsen, Falch Christiansen, Hartling and Trap-Jensen, 1977) ion exchangers.

Measurement of ionized magnesium

The first attempt to measure ionized magnesium was made by Nordbo (1939) who used an indicator dye. Baum and Czok (1959) then developed an enzymatic method but obtained very variable results. Walser (1960) used the indicator eriochrome black-T in a spectrophotometric method for estimating ionized magnesium. The change in absorbancy due to change in the relative concentrations of the two absorbing species magnesium-dye and H_2-dye at 530 nm, was directly proportional to the ratio $K/[Mg^{++}]^n$, where n is the binding ratio of the magnesium-dye complex and K the equilibrium constant. This method has not been used except by the original author. To eliminate interference from plasma proteins ultrafiltration is required. Relatively large volumes of tris buffer have to be added to the ultrafiltrate to control pH, this may alter equilibria present. The complications involved in this method make it of questionable value for routine studies.

Low capacity ion exchange strips have been used for measuring ionized calcium and magnesium (Frizel, Malleson and Marks, 1967; Heaton, 1967). The principle of this type of method is the adsorption of cations by ion exchange materials in proportion to their relative concentrations and in proportion to their relative affinities for the ion exchanger. The capacity of the ion exchanger is 1-2% of the total plasma calcium, ensuring that minimal derangement of existing equilibria is produced.

Frizel et al (1967) used an ion exchanger containing aryl sulphonic acid groups. They showed that magnesium and calcium were taken up by the ion exchange strip in proportion to their concentrations. They also found that increasing the concentrations

of sodium or potassium in the standard depressed the adsorption of calcium, or magnesium, by the ion exchange strips. When citric acid was added, the uptake of calcium and magnesium by the strips was reduced. Heaton (1967) also used ion exchange strips and showed that the uptake of magnesium was proportional to its concentration in standard solutions and inversely proportional to the concentration of interfering cations.

Ion exchange methods seem to offer a relatively simple approach to the measurement of ionized magnesium which has the advantage of minimal sample handling thus reducing the risk of pH changes. The work of Heaton (1967) is widely quoted (for example: Achilles, Scheidt, Hoppe and Cumme, 1977; May, Linder and Williams, 1977; Bird and Maguire, 1978; Flatman and Lew, 1980; Speich, Bosquet and Nicolas, 1981), possibly because of the low variation in his results and the excellent agreement with the results of Walser (1961).

Complexed calcium and magnesium

This is normally the smallest component of plasma calcium and magnesium. It is usually determined as the difference between ultrafiltrable and ionized calcium or magnesium; the compounding of estimation errors and the small size of this fraction make it is very difficult to study. The main complexes normally present in plasma are those formed by citrate, phosphate (Walser, 1961a) and bicarbonate (Greenwald, 1941; Neuman, Morrow, Toribara, Casarett, Mulryan and Hodge, 1956). Whilst this fraction normally constitutes only a small proportion of the total plasma calcium and magnesium concentrations, during infusions of calcium and magnesium binding

ligands the size of this fraction may become significant.

Speich, Bosquet and Nicolas (1981) described a method for measuring complexed magnesium. The ionized and protein bound magnesium were claimed to be adsorbed by a cation exchange resin. The residual magnesium was assumed to be the complexed form which was then measured by atomic absorption spectrophotometry. Since this method involves the removal of all the ionized magnesium from the sample, it would seem that in maintaining equilibrium complexed magnesium would dissociate, so releasing magnesium to be adsorbed by the resin. This would make measurement of the true concentration of the complexed form in the original sample unlikely.

Protein bound calcium and magnesium

McLean and Hastings (1934b) used their ionized calcium assay to study calcium binding. They found that in protein containing fluids such as ascitic, pleural or oedema fluids, serum or artificial protein solutions, the ionization of calcium followed the law of mass action. They were able to use their methods to determine equilibrium constants for the calcium-protein reaction. No such studies have been made in the case of magnesium, though attempts were made to determine magnesium-protein equilibrium constants using ultrafiltration (Copeland and Sunderman, 1952). Since ionized magnesium was not measured, these constants are likely to be less accurate.

In serum, albumin is responsible for binding 90% of the non-diffusible calcium (Pedersen, 1972d; Tofaletti, Savory and Gitelman, 1977) and all the protein bound magnesium (Cummings, Kuff

and Sober, 1968). The nature of the bonds formed between calcium and serum albumin has received detailed study (Pedersen, 1971b, 1972a,b,c&d). Calcium and magnesium have been shown to compete for protein binding sites on the serum albumin molecule (Carr 1955; Pedersen 1972b&d), the second author also demonstrating competition between H^+ and calcium ions. In vivo studies in rats have demonstrated a strong negative correlation between ionized plasma calcium and plasma pH. This was probably, at least in part, a result of changes in protein binding (Girndt, Henning and Delling, 1979).

Determination of diffusible calcium and magnesium

Many techniques have been used for this determination, basically they all involve either ultrafiltration through a semi-permeable membrane (Rawson and Sunderman, 1948; Robertson and Peacock, 1968; Pedersen, 1972b) or ultracentrifugation (Chanutin, Ludwig and Masket, 1942; Ludwig, Chanutin, and Masket, 1942; Breen and Freeman, 1961; Cummings et al, 1968). Most methods of ultrafiltration use either positive pressure (Rose, 1957; Robertson and Peacock, 1968) or centrifugation (Pedersen, 1971b) to induce flow of ultrafiltrate through the semi-permeable membrane. Positive pressure has the disadvantage of altering pCO_2 and thus plasma pH and protein binding of calcium and magnesium.

Various types of membrane filters have been used including collodion membranes (Tschimber and Tschimber, 1924; Greenberg and Greenberg, 1931), visking tubing (Rawson and Sunderman, 1948; Pedersen, 1971b), cuprophan membranes (Le Grimellec, Poujeol and de Rouffignac, 1975) and Amicon "Centriflo" membranes (Holley and

Evans, 1977). The type of filter used seems to be insignificant providing the pores are small enough to prevent the passage of protein molecules. Estimates of pore size in visking tubing may be made by measuring the increased resistance to flow that occurs with increased molecular weight of solutes. Studies suggest pore radii of 1.3-2.3 nm (Renkin, 1954; Durbin, 1960) which is sufficiently small to exclude all plasma proteins. Several factors require consideration in the assessment of a method of determining diffusible calcium and magnesium. These factors all seem to act through Donnan (Greenberg and Greenberg, 1931) or pH effects (Robertson et al, 1968).

Donnan (1924) demonstrated that when non-diffusible substances, such as negatively charged protein molecules, are contained within a semi-permeable sac, as a result of electrical and concentration gradients diffusible cations move in whilst anions move out. When these opposing forces equilibrate, a negative potential is present within the sac whose magnitude is dependent on the Nernst equation. When ultrafiltration of plasma takes place, the removal of protein free filtrate increases the protein concentration and enhances the Donnan effect. It is therefore important that excess ultrafiltrate should not be removed from the sample.

Since calcium, magnesium and H^+ compete for the same binding sites on the albumin molecule (Carr, 1955; Pedersen, 1971a,1972a&d) it is important that blood pH is carefully controlled during the procedure. Temperature also effects blood gas equilibria and pH and so is also likely to require careful control.

Ultracentrifugation can avoid the Donnan shifts associated with the use of semipermeable membranes but requires the use of sophisticated equipment and necessitates pH control during

prolonged periods of centrifugation. Given careful control, it would seem that ultrafiltration can provide a reasonable estimate of diffusible calcium and magnesium in plasma.

The extent to which plasma ultrafiltrates resembles the composition of glomerular filtrate is not absolutely clear. It is likely that differences exist since the glomerulus is permeable to some plasma proteins (Dirks, Clapp and Berliner, 1964) whilst the pore size of most ultrafiltration systems is usually considerably smaller and so prevents the passage of all proteins.

II. The renal handling of calcium and magnesium

Calcium

Clearance studies have shown that the renal handling of calcium involves glomerular filtration with subsequent reabsorption of most of the calcium by the renal tubule (Chen and Neuman, 1955a). Micropuncture studies showed that the concentration of calcium in the glomerular ultrafiltrate is approximately 60-70% of total plasma calcium (Lassiter, Gottschalk and Mylle, 1963; LeGrimellec, Poujeol and De Rouffignac, 1975). The second study did not find a significant correlation between plasma ultrafiltrable and glomerular filtrate calcium concentration. It was not certain whether this merely reflected methodological variation, or whether it was the result of genuine differences.

Lassiter *et al* (1963) were the first to use micropuncture to study calcium transport in different segments of the nephron. Of the filtered calcium, 60% was absorbed in the proximal tubule along

with sodium and water. Murayama, Morel and Le Grimellec (1972) found that the proximal tubule was highly permeable to calcium since net reabsorption was only 1/3 of the unidirectional flux.

In the loop of Henle approximately 25% of filtered calcium is reabsorbed (Lassiter et al, 1963). Rocha, Magaldi and Kokko (1977) showed that the calcium permeability of all segments of the loop of Henle was too low to allow calcium reabsorption by passive means. Furthermore they demonstrated that in this segment, the thick ascending limb was the primary reabsorptive site.

In the distal tubule approximately 10% of filtered calcium is reabsorbed (Lassiter et al, 1963). Since reabsorption at this site occurs against the trans-tubular potential (Clapp, Rector and Seldin, 1962) and against a large concentration gradient (Lassiter et al 1963), it is likely that reabsorption involves active transport.

Magnesium

Magnesium, like calcium, is filtered by the glomerulus and then undergoes reabsorption in the renal tubule. Approximately 70-80% of total plasma magnesium is filtered by the glomerulus (Le Grimellec, Poujeol, and De Rouffignac, 1975; Brunette and Crochet, 1975). The concentration of magnesium in glomerular filtrate thus appears to be similar to values determined in plasma ultrafiltrates (Walser, 1961a).

Magnesium is partially reabsorbed in the proximal tubule. Brunette, Wen, Evanson and Dirks (1969) found that in the dog, proximal tubular magnesium absorption appeared to be isotonic and followed sodium and water. Other studies have since shown that

although magnesium reabsorption occurs, the proximal tubule is relatively impermeable to magnesium and so the ratio of tubular fluid to plasma ultrafiltrate magnesium concentration rises along this segment. This was demonstrated by micropuncture studies in the rat (Morel, Roinel and Le Grimellec, 1969) and by studies of ^{28}magnesium permeability (Brunette and Aras, 1971). The distal convoluted tubule is not a major site of magnesium transport. Limited reabsorption occurs in this segment and is concentration dependent (Quamme and Dirks, 1980).

The work of Morel et al (1969) indicated that 50-60% of filtered magnesium was reabsorbed in the loop of Henle (De Rouffignac, Morel, Moss and Roinel, 1973). Brunette, Vigneault and Carriere (1978) found that at the tip of the loop of Henle magnesium concentration was considerably higher than in the vasa recta. This suggested that magnesium absorption was not occurring to any great extent in the descending limb. During preliminary experiments in which rabbit cortical thick ascending limbs were perfused in vitro, Shareghi and Agus (1979) found that magnesium was absorbed from the lumen suggesting that this portion of the ascending limb was the important area of magnesium reabsorption.

Some factors influencing the renal handling of calcium and magnesium

Endocrine influences

The possible involvement of parathyroid hormone (PTH) in the control of renal calcium excretion was first suggested by an acute rise in urinary calcium following parathyroidectomy of rats (Talmage and Kraintz, 1954). Since hypercalciuria occurred despite decreased plasma calcium, it seemed likely that PTH had been promoting tubular reabsorption of calcium. Kleeman, Rockney and Maxwell (1958) subsequently demonstrated reduced calcium clearance in man following administration of parathyroid extract. The reduction was constant over the wide range of calcium clearances induced by calcium chloride administration.

In the dog parathyroidectomy also induces hypercalciuria (Kleeman, Bernstein, Dowling and Maxwell, 1960). Similarly, PTH reduces calcium clearance in the dog (Widrow and Levinsky, 1962), the rat (MacIntyre, Boss and Troughton, 1963) and in man (Edwards and Hodgkinson, 1965). Clearance and stop-flow[1] studies suggest that PTH acts mainly at a distal site (Widrow and Levinsky, 1962;

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[1] Stop-flow studies are performed by inducing rapid diuresis, usually using mannitol. Clamping the ureter stops or slows glomerular filtration and urine flow through the nephron allowing time for the nephron segments to alter solute concentrations. On release of the clamp glomerular filtration rate and urine flow rapidly rise to their former values and the altered urine is rapidly flushed into the ureter with minimal alteration during its passage through subseqent tubule segments. The urine is collected in serial samples, early samples being from the distal tubular segments and later samples from the proximal segments. Relating the effects on solutes to the simultaneous measurements of sodium, inulin and para-aminohippuric acid provides information regarding the tubular transport of these solutes. Despite the assumptions involved in the method, it has proved to be useful in the dog.

Massry, Coburn, Chapman and Kleeman, 1967). Micropuncture studies have confirmed that in the dog PTH has a selective action on calcium reabsorption at or beyond the distal tubule (Sutton, Wong and Dirks, 1976). Evidence for an action in the proximal tubule and at a site between the proximal and distal tubule has also been produced (Harris, Burnatowska, Seely, Sutton, Quamme and Dirks, 1979).

PTH seems to have an effect on renal magnesium handling similar to that on calcium. MacIntyre _et al_ (1963) showed that PTH depressed magnesium excretion. Massry, Coburn and Kleeman (1969) found that during magnesium infusion the fractional excretion of magnesium was reduced by parathyroid extract. Unfortunately many studies are difficult to interpret because of the close relationship between the tubular reabsorption of calcium and magnesium.

Although calcitonin seems to be less important in calcium homeostasis, it has been found to enhance calcium excretion (Ardaillou, Vuagnat, Milhaud and Richet, 1967; Singer, Woodhouse, Parkinson and Joplin, 1969).

Anions

Anions present in plasma and renal tubular fluid significantly affect the renal handling of calcium and other ions. The effects of ligands on aspects of calcium and magnesium metabolism will be considered in greater detail in a later section. Sodium citrate increases the rate of excretion of calcium (Gomori and Gulyas, 1944) as does EDTA (Spencer, Vankinscott, Lewin and Lazlo, 1952). Sulphate causes both calciuresis and magnesuresis (Wolf and Ball,

1950). Gluconate increases the renal clearance of calcium (Bernstein, Kleeman, Cutler, Dowling and Maxwell, 1962). The calciuretic response to many anions is primarily a result of the formation of complexes which are unavailable for renal tubular reabsorption. Additionally, the formation of complexes may alter plasma ultrafiltrable calcium and thus the rate of filtration of calcium by the glomerulus. This latter effect has been demonstrated with EDTA (Soffer and Toribara, 1961).

Anions with a valency greater than one probably also depress tubular reabsorption by increasing the negative intratubular potential and thus the opposing electrical gradient against which cations must be absorbed (Clapp *et al*, 1962).

Hydrogen ion

Evidence suggesting that acidosis alters the renal handling of calcium was first produced many years ago. Givens and Mendel (1917) and Lamb and Evvard (1919) demonstrated that administration of mineral acids augmented renal calcium excretion. The sheep was found to develop a post prandial acidosis accompanied by hypercalciuria and hypermagnesuria (Stacy, 1969). Stacy and Wilson (1970) showed that hypercalciuria could be produced in the sheep by respiratory or metabolic acidosis even when the filtered calcium load was falling, this strongly implicates a direct inhibitory action of acidosis on the renal tubular reabsorption of calcium.

Sutton Wong and Dirks (1979) performed micropuncture and clearance studies in rats. Their studies suggested that H^+ ions depressed calcium reabsorption in the loop of Henle and in the terminal nephron.

The inverse relationship between the renal tubular reabsorption of calcium and magnesium

The close relationship between calcium and magnesium excretion has been recognised for many years. Mendel and Benedict (1909a&b) showed that infusion of calcium or magnesium chloride caused an increase in the excretion of both ions. Wolf and Ball (1949) showed that calcium had a magnesuric effect. Massry, Coburn and Kleeman (1969) performed renal clearance studies in dogs whilst infusing calcium or magnesium chlorides. The tubular reabsorption of magnesium increased in proportion to plasma magnesium and reached a maximum at high plasma concentrations. Infusion of calcium salts or saline diuresis depressed the magnesium tubular maximum. Coburn, Massry and Kleeman (1970) confirmed the effect of calcium chloride on magnesium excretion and also showed that the effect occurred even when glomerular filtration rate (GFR) was markedly reduced.

Evidence has been produced that this competition occurs in the distal segment of the nephron. Samiy, Brown, Globus, Kessler and Thompson (1960) performed stop flow studies in dogs (see footnote p 14). After release of the ureteral clamp, calcium and magnesium were measured in sequentially collected urine samples. The concentrations of both ions reached minima in the same samples, suggesting that magnesium and calcium both underwent reabsorption in the same portion of the renal tubule. Quamme and Dirks (1980) carried out micropuncture and single nephron perfusion studies. They showed that hypermagnesaemia caused a reduction in the reabsorption of both calcium and magnesium from the loop of Henle.

In view of the renal interaction between these two ions, it might be expected that infusion of calcium would cause hypomagnesaemia and vice versa. The hypocalcaemic effect of magnesium infusions has been demonstrated in man, dogs and cats (Kelly, Cross, Turton & Hatcher, 1960; Keměny, Boldizăr and Pethes 1961; Pors Nielsen, 1970). No evidence has been produced for a hypomagnesaemic action due to calcium, possibly because the relatively large variation normally occurring in plasma magnesium concentrations would render this more difficult.

III. Effects of ligands on calcium and magnesium metabolism

Ligands are molecules or ions which form bonds with metallic ions through their spare valency electrons. When such bonds form the resulting complex has properties which differ from those of its constituents. A chelate is a special case of a ligand which binds the metal ion at two or more points thus forming a heterocyclic ring. Depending on the number of bonds formed, chelates may be bidentate, tridentate or polydentate. The metal ions bound to chelates tend to be largely unavailable because of the extensive shielding and the strong bonds which form. The extent to which the complexed metal ions will be unavailable will depend on the degree of covalency of the bonds formed and the degree of shielding afforded by the ligand as compared to the hydrated cation. The nature and stability of the chemical bonds formed has been reviewed by Moeller and Horwitz, (1960).

Endogenous ligands and chelates are widespread in nature. Their ability to bind cations is an important determinant of biological

function. Substances such as chlorophyll, haem and vitamin B_{12} depend on chelate formation for their desired properties. Many enzymes require metal ions for their activity. In some enzymes the metal is strongly bound by protein, but in others only very weak bonds are formed. Magnesium is required by the important group of enzymes involved in the transfer of phosphate groups, including phosphatases and the enzymes involved in the reactions of ATP. It is also involved in many decarboxylation reactions (Wacker and Vallee, 1964).

The importance of exogenous ligands in pharmacology is widely recognised. Various aspects of this subject have been extensively reviewed (Chenoweth, 1956; Johnson and Seven, 1961; Pullman, Lavender and Foreland, 1963; Levinson, 1980). For example, the ability of EDTA, dimercaprol and penicillamine to form chelates is central to their use in the treatment of heavy metal poisoning. They act by making the metal ions unavailable to tissues and by enhancing their renal excretion.

The calciuretic and magnesuretic effects of ligands, primarily resulting from inhibition of renal tubular reabsorption, has already been discussed. The formation of calcium complexes by ligands often involves the displacement of H^+ ions and so such reactions are pH dependent. They are also dependent upon ionic strength. It is to be expected therefore that the equilibrium constants, and thus the degree of ligand binding, will vary along the renal tubule as changes in pH and ionic strength occur. These changes, occurring at the main sites of tubular reabsorption, influence the degree to which the presence of ligand will alter the tubular reabsorption of calcium and other cations and may well result in non-stoichiometric binding of calcium and magnesium.

As might therefore be expected, the increment in calcium excretion associated with sulphate diuresis is invariably less than predicted (Walser, 1961c). In the case of powerful calcium complexing agents such as EDTA it might be expected that urinary EDTA would be closely related to the increment in urinary calcium. During EDTA infusion in man studies have indicated that the increment in urinary calcium excretion is only 60-70% of that predicted on the basis of stoichiometric binding (Spencer et al, 1952; Parfitt, 1969). Rubin and Lindenblad (1956-7) showed a similar effect in the rat and produced evidence that this effect might be dependent on urinary pH. In view of the massive changes in EDTA-calcium binding accompanying pH changes, it is quite feasible that the deficit in urinary calcium during EDTA infusion may be due to pH changes along the renal tubule causing incomplete calcium binding.

Since the binding constants of citrate for calcium and magnesium are similar (Walser, 1961b; Meyer, 1974), large infusions of citrate, by enhancing excretion, might be expected to lower the plasma ionized concentrations of both calcium and magnesium. This was demonstrated during infusions of acid citrate dextrose in dogs (Killen, Grogan, Gower and Collins, 1971).

Two of the substances studied in this thesis, EDTA (ethylene diamine tetraacetic acid) and EHDP (ethane-1-hydroxy-1,1-diphosphonic acid) are known to have calcium binding properties. The third, calcium borogluconate, was investigated as it was considered likely to complex calcium.

Exogenous ligands: 1. Ethylene diamine tetraacetic acid (EDTA)

EDTA is a chelate which is widely used for complexometric titrations since at high pH it chelates calcium and magnesium stoichiometrically (Schwartzenbach, 1957). The great stability of the chelates formed may be related to the number of bonds formed with the metal ion (figure 1.1). EDTA binds the heavy metals most strongly. However in the environment of blood, calcium is the predominantly bound cation. Once virtually all the calcium is bound then free EDTA will chelate magnesium (Shwartzenbach, 1957). As with all ligands, competition between H^+ and metal cations occurs. The relationship shown in figure 1.2 indicates the massive changes in binding following pH changes (after Martell and Calvin, 1952; quoted by Rubin and Lindenblad, 1956-7).

The calcium-EDTA chelate is very useful for the treatment of heavy metal poisoning. The copper chelate is used in treatment and prophylaxis of copper deficiency in cattle and sheep.

EDTA is not metabolized by the rat (Foreman, Vier and Magee, 1953). Early reports, based on blood clearance calculations, (Foreman _et al_, 1953) suggested EDTA was excreted by both glomerular filtration and tubular secretion. Clearance studies in dogs subsequently showed that it was excreted by glomerular filtration alone (Forland, Pullman, Lavender and Aho, 1966) and may be used to measure glomerular filtration rate. In the rat 95-98% of EDTA is eliminated after six hours (Foreman _et al_, 1953).

Soffer and Toribara (1961) infused EDTA into human subjects and measured total and ultrafiltrable calcium. EDTA had no effect on total calcium but increased calcium chelate concentration in plasma as measured by the increase in ultrafiltrable calcium and the

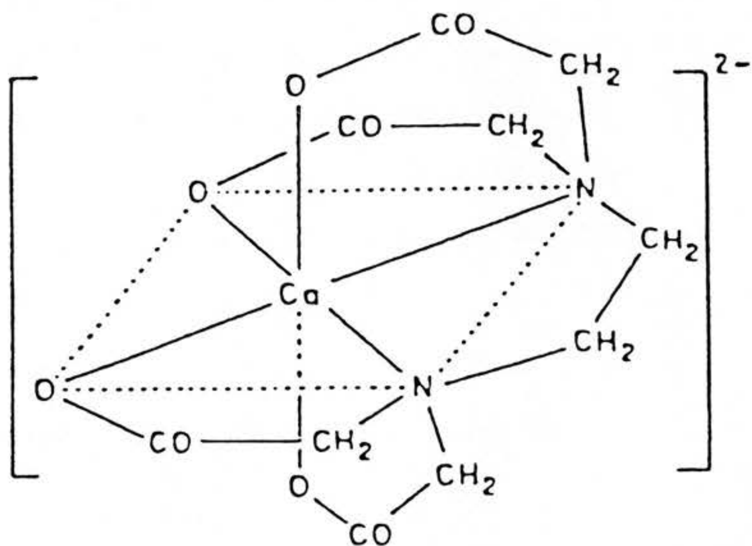


Figure 1.1 Spatial representation of the bonding of calcium by ethylene diamine tetra-acetic acid (from: Martell, 1960).

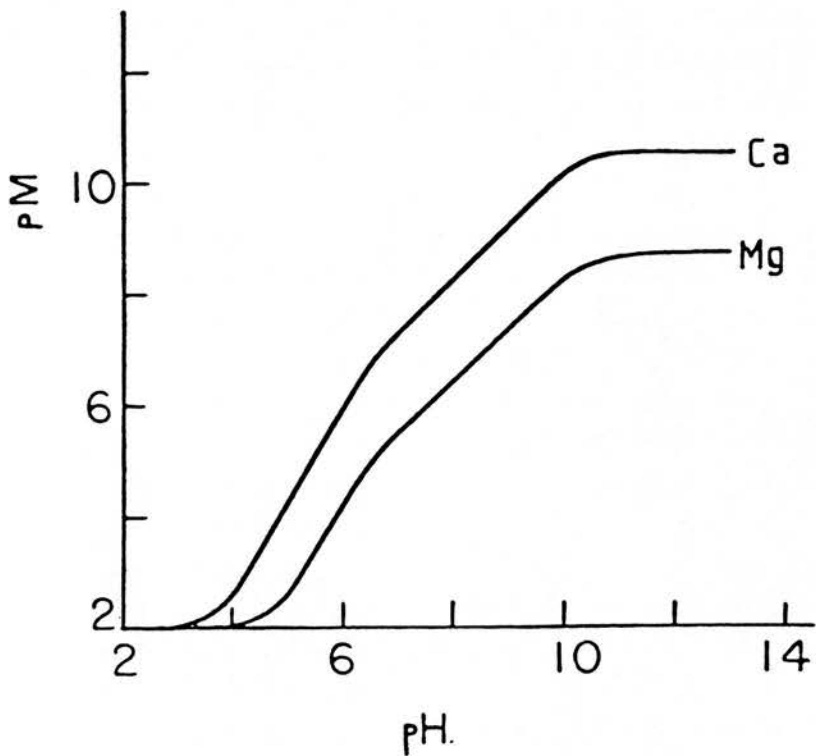


Figure 1.2 The effect of pH on the -ve log [cation] (pM) in the presence of EDTA (from: Martell and Calvin, 1952).

decrease in calculated ionized calcium. This effect must have been due to the presence of EDTA chelated calcium in plasma. Parfitt (1969) infused Na_2EDTA into man and found that ionized plasma calcium fell during the infusion, then rapidly rose when infusion ceased. Total plasma calcium, in contrast, was unchanged. Again this indicates an increase in bound calcium in blood.

Many workers have demonstrated that EDTA infusion is accompanied by hypercalciuria (Spencer, Vankinscott, Lewin and Lazlo, 1952; Holland Danielson and Sahagian-Edwards, 1953; Chen and Neuman, 1955a; Rubin and Lindenblad, 1956-7; Levitt, Halpern, Polimero, Sweet and Gribetz, 1958). Stop-flow studies have demonstrated a distal minimum calcium concentration in samples corresponding to the distal tubule (Howard, Wilde and Malvin, 1959). This distal minimum is obliterated by EDTA (Grollman, Walker, Harrison and Harrison, 1963) indicating depression of calcium reabsorption in the distal tubule.

EDTA infusions seem to be associated with an increase in the bound plasma calcium and a decrease in ionized plasma calcium. Calciuresis results from the failure of the tubules to reabsorb calcium-EDTA complexes. No effects on magnesium seem to have been reported in the literature.

Exogenous ligands: 2. Calcium borogluconate

Dryerre and Greig (1935) were the first to advocate the use of calcium borogluconate solution for the treatment of post-parturient hypocalcaemia in cattle. It was recommended for its great solubility and non-irritant nature. They also showed that the crystallised solid was a distinct chemical compound. Macpherson and Stewart (1938) confirmed and extended these observations, demonstrating that it contained 2 moles of borate per mole of gluconate. However they also concluded that in aqueous solution the boric acid seemed to exist as a free entity unassociated with calcium gluconate.

Although there is no published evidence that borogluconate complexes calcium, reports suggest that gluconate does. The administration of calcium gluconate to patients undergoing cardiac surgery was considerably less effective in raising blood ionized calcium than was the administration of equivalent amount of calcium chloride (White, Goldsmith, Rodriguez, Moffit and Pluth, 1976). It was suggested that this effect was due to the formation of calcium-gluconate complexes and that metabolism of the anion might be required before release of free calcium ions.

Evidence that gluconate alters the renal handling of calcium was produced by Howard Wilde and Malvin (1959) who performed stop-flow studies in the dog (see footnote page 14). Calcium gluconate infusion greatly increased the minimum calcium concentration in early samples of urine collected after release of the ureteral clamp, infusion of calcium chloride did not. It appeared that calcium gluconate, but not calcium chloride, prevented the reabsorption of calcium from within the distal segment of the renal

tubule. Distal sodium and potassium reabsorption also seemed to be depressed since the gluconate ion increased the free flow concentrations of calcium, sodium and potassium. These results were explained on the basis of a non-specific electrostatic attraction produced by the large non-diffusible gluconate anions present in the tubular fluid. Thus reabsorption of all cations was depressed. However it seems likely that, as with other calcium complexing agents such as EDTA, these renal effects were partially due to the formation of calcium complexes.

Other workers demonstrated an increased clearance of calcium during calcium gluconate infusion (Bernstein, Kleeman, Cutler, Dowling and Maxwell, 1962). Dibona (1971) used micropuncture to collect proximal tubular fluid from rats during intravenous infusions of calcium chloride or calcium gluconate. Neither solution affected proximal tubular calcium reabsorption whereas only gluconate enhanced calcium excretion. This evidence supported the stop-flow studies of Howard et al (1959) and was taken to indicate inhibition of distal tubular reabsorption. Evidence relating to the binding of magnesium by either gluconate or borogluconate was not found in the literature.

Exogenous ligands: 3. Ethane-1-hydroxy-1,1-diphosphonic acid (EHDP)

Condensed phosphates are thought to play an important role in mineral metabolism. Since the P-O-P bonds of condensed phosphates are rapidly hydrolysed *in vivo*, the diphosphonates (containing a stable P-C-P group) were developed to assist investigations into the role of condensed phosphates in mineral metabolism. EHDP belongs to this group of compounds, it is known to complex calcium (Carroll and Irani, 1968; Grabenstetter and Cilley, 1971). In order to appreciate the significance of the diphosphonates it is instructive first to consider evidence relating to condensed phosphates.

Condensed phosphates in vitro

Condensed phosphates have an inhibitory action on the precipitation of calcium carbonate (Buehrer and Reitmeier 1940), calcium sulphate (Reitemeier and Ayers, 1947) and calcium oxalate (Fleisch and Bisaz, 1964). Detailed investigations of this phenomenon have been carried out with strontium sulphate (Miura, Otani, Kodama and Shinagwa, 1962) and have shown the inhibitory action to be due to coating of the crystal nuclei with a mono-molecular layer.

It is known that, with respect to calcium and phosphate, the extracellular fluid is supersaturated. The concentrations of calcium, phosphate and OH^- are high enough to support the growth, but not the initial formation, of bone hydroxyapatite crystals ($Ca_5OH(PO_4)_3$). Fleisch and Neuman (1961) demonstrated that collagen could act as a nucleus for the initiation of crystal formation. Plasma ultrafiltrates were then shown to contain

substances which inhibited the precipitation of hydroxyapatite, as measured by the minimum [Ca] x [P] product initiating preciptation. Several condensed phosphates, including pyrophosphate, also had this inhibitory action. The inhibitory action of plasma ultrafiltrates was destroyed by alkaline phosphatase, the implication being that this might be due to destruction of condensed phosphate (Fleisch et al, 1961).

Since collagen is not normally calcified this work raises the interesting possibility that condensed phosphates may play an important role in both the initiation and prevention of calcification in normal tissues. If this is the case and if pyrophosphate is the condensed phosphate in question, then the control of the tissue concentration of these substances and thus the control of calcification may be mediated by pyrophosphate and pyrophosphatases.

Pyrophosphate and pyrophosphatase in vivo

Pyrophosphate has been detected in urine (Fleisch and Bisaz, 1962), bone (Perkins and Walker, 1958) and teeth (Bisaz, Russell and Fleisch, 1968). Russell, Bisaz, Donath, Morgan and Fleisch, (1971) found that plasma pyrophosphate was raised in hypophosphatasia, a hereditary condition characterised by bone abnormality and deficiency of alkaline phosphatase. Alfrey and Ibels (1977) found high levels of pyrophosphate in the visceral calcified deposits occurring in some patients with chronic renal failure. Thus it may be that although pyrophosphate prevents precipitation of calcium phosphate from solution, once precipitation has occurred dissolution is also inhibited.

Several enzymes having pyrophosphatase activity have been found. Cartier and Picard (1955) demonstrated the presence of an ATP-pyrophosphatase present in sheep embryo cartilage. In the costal cartilage of young rats Alcock and Shils (1969) found a neutral pyrophosphatase, its concentration increased in proportion to the rate of uptake of ^{45}Ca suggesting a role in bone calcium uptake. More recently, Morgan, Monod, Russell and Fleisch (1973) demonstrated an acid phosphatase possessing pyrophosphatase activity. Its production by bone was inhibited by dichloromethylene diphosphonate. Alkaline phosphatase also has pyrophosphatase activity at neutral pH.

In vivo, pyrophosphate can prevent vitamin D_3 induced aortic calcification (Schibler, Russell and Fleisch, 1968) and experimentally induced skin calcinosis (Gabbiani, 1966). It has no effect on bone *in vivo*, but prevents bone calcification in tissue culture (Fleisch, Strauman, Schenk, Bisaz and Allgower, 1966). This failure may be due to the high levels of pyrophosphatase activity in bone.

The quoted literature suggests many relationships between pyrophosphate and mineral metabolism. However the significance of these findings is uncertain. Since pyrophosphate is rapidly inactivated *in vivo*, compounds were developed which were resistant to hydrolysis yet retained pyrophosphate-like activity. Imidophosphates are compounds containing P-N-P linkages, in contrast to the P-O-P linkage of pyrophosphate. These substances have been shown to have similar activity to pyrophosphate *in vitro* (Robertson and Fleisch, 1970), but are claimed to have no activity *in vivo* (Fleisch, 1975). Diphosphonates contain a P-C-P linkage, they are active *in vivo* and have been extensively investigated.

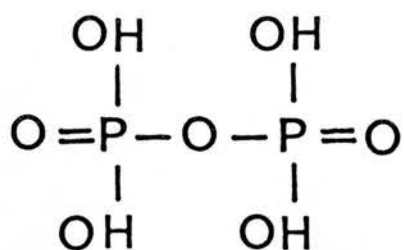
Diphosphonates

The two diphosphonates which have received the most detailed study, EHDP (ethane-1-hydroxy-1,1-diphosphonate) and dichloro-methylene diphosphonate, are compared with pyrophosphate in figure 1.3. This group of compounds has been the subject of a number of reviews (Fleisch, Bisaz, Care, Müllbauer and Russell, 1969; Russell and Fleisch, 1975; Fleisch, 1975; Fleisch, 1980).

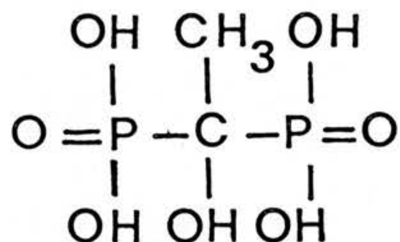
The absorption of EHDP from the gastro-intestinal tract is very poor. Approximately 1-8 % is absorbed in man, rhesus monkeys, rats and rabbits, and 10-12 % in the dog. Absorption is slightly higher in puppies and weanling rats (Michael, King and Wakim, 1972; Recker and Saville, 1973).

Michael *et al* (1972) found that in a number of different species, 50% of an absorbed or injected dose of EHDP was rapidly excreted in the urine. The remainder was taken up by bone and had a half life of 12 days in the rat. The clearance of ^{99m}Tc-EHDP, as a fraction of inulin clearance, was found to be 27.3% in the dog (Hughes Davies, Bassingthwaighte, Knox and Kelly, 1977). Studies using isotopically labelled EHDP indicate that it is not metabolized (Michael *et al*, 1972).

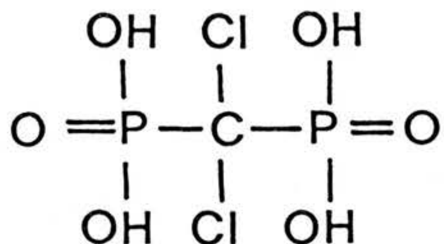
In vitro, diphosphonates have a number of actions similar to the effects of pyrophosphate. They probably inhibit the precipitation of calcium phosphate from solution and the conversion of amorphous calcium phosphate to crystalline hydroxyapatite by binding onto the crystal surface (Francis, 1969; Fleisch, Russell and Francis, 1969). EHDP produces a calcium fraction in hydroxyapatite suspensions which is non-ultrafiltrable, non-sedimentable and which



Pyrophosphoric acid



Ethane-1-hydroxy-1,1-diphosphonic acid



Dichloromethylene diphosphonate

Figure 1.3 Comparison of the structure of two diphosphonates with pyrophosphoric acid.

does not exchange with ^{45}Ca (Robertson, Morgan, Fleisch and Francis, 1972). These effects may be the result of EHDP coating the crystal nuclei in a manner analagous to that described by Miura et al (1962). In addition to these properties, common to both pyrophosphate and diphosphonates, diphosphonates also show some properties which may be specific.

Several studies have been carried out which show that diphosphonates complex calcium. Carroll and Irani (1968) performed potentiometric studies and determined dissociation constants for the formation of 1:1 calcium-EHDP complexes. Grabenstetter and Cilley (1971) used both pH and complexometric titrations to investigate calcium-EHDP binding. Large aggregates were produced which they termed poly-nuclear complexes. The maximum EHDP:calcium binding ratio was found to be 1.75:1. These workers used computer curve fitting techniques to determine the calcium-EHDP ratios which best fitted their data and found that a combination of the ratios of 1:1, 3:2, 4:3, and 7:4 gave the best fit. Wiers (1971) also demonstrated the formation of large complexes which were resistant to precipitation. Their size was of the order of 10^4 Daltons and their approximate radius 2.6 nm. Bisaz et al (1978) showed that the ^{99m}technetium complex of EHDP, ^{14}C-EHDP, ^{14}C-dichloro-methylene diphosphonate, and ^{32}P-pyrophosphate were partially non-ultrafiltrable in plasma. They used ultrafilters with a nominal cut off point of 50,000 Daltons which suggests either protein binding or the formation of very large complexes.

Despite the detailed studies that have been made of the physico-chemical interactions between EHDP, hydroxyapatite and calcium, the complexation of calcium per se has received relatively little study. The work of Grabenstetter and Cilley (1971) and Wiers

(1971) has been mentioned, however it is difficult to extrapolate from those results to the physiological situation since the studies were made at pH 11 and many of the experiments were performed using micro-molar concentrations of free calcium.

Biological actions of diphosphonates

Rats given large amounts of vitamin D developed aortic and renal calcification which could be prevented by various diphosphonates given *per os* or by subcutaneous injection (Fleisch, Russell, Bisaz, Mullbauer and Williams, 1970). EHDP, dichloro-methylene diphosphonate and methylene diphosphonate were the most effective ones tested. In contrast pyrophosphate, which must administered parenterally, only inhibited aortic calcification.

If Ringer solution is placed in the frog bladder, removal of calcium with chelating agents greatly enhances water permeability of the membrane. This effect has been used to study calcium binding *in vitro* (Shakhmatova, Kabachnik, Medev′ and Natochin, 1982). EHDP was shown to have a large effect on water permeability. Though not as effective as EDTA, it was more effective than the other diphosphonates studied.

The renal effects of diphosphonates

EHDP appears to prevent the renal hydroxylation of 25 hydroxycholecalciferol (Hill, Lumb, Mawer and Stanbury, 1973). This conclusion was reached following the demonstration that EHDP depressed calcium absorption and that this effect was reversed by 1,25 dihydroxycholecalciferol but not by huge doses of cholecalciferol or 25 hydroxycholecalciferol. The defect develops almost immediately and so seems to be independent of the mineralisation defect which takes several days to develop. EHDP is known to increase plasma phosphate in man due to an increase in the maximal tubular reabsorption of phosphate (Walton, Russell and Smith, 1975). This is not accompanied by significant changes in GFR or plasma or urinary calcium. Large doses of EHDP (20 mg kg^{-1} s.c., Goulding and Cameron, 1977; 40 mg kg^{-1} s.c., Gasser et al, 1972) caused increases in both urinary and plasma calcium whilst dichloro-methylene diphosphonate had no effect.

The effects of diphosphonates on bone

Diphosphonates have a very strong affinity for bone (Michael et al, 1972). This property has enabled the use of ^{99m}technetium labelled EHDP for producing bone scans (Subramanian, McAfee, Blair, Mehter and Connor, 1972). EHDP is now widely utilised for this purpose.

EHDP administered to young growing rats at 4 mg kg^{-1} depressed the rate of bone resorption whilst having little effect on formation (Gasser, Morgan, Fleisch and Richelle, 1972). At 40 mg kg^{-1} the effect was greater and bone formation was also depressed. Dichloro-methylene diphosphonate, by comparison, has a more

specific effect on resorption. In isolated mice calvaria, parathyroid extract induced bone resorption was prevented by dichloro-methylene diphosphonate or methylene diphosphonate (Fleisch, Russell and Francis, 1969). Furthermore, the hypercalcaemic action of parathyroid extract in parathyroidectomised rats was prevented by dichloro-methylene diphosphonate or methylene diphosphonate.

During dichloro-methylene diphosphonate (4 mg kg^{-1}) administration to pre-parturient cattle, bone catabolism seemed to be depressed as evidenced by reduced urinary hydroxyproline and micro-radiography (Yarrington, Capen, Black, Re, Nagode and Geho, 1977a). The response of plasma calcium concentration to stress induced by EDTA infusion can provide an index of an animal's ability to mobilise bone calcium. In these experiments the animals treated with dichloro-methylene diphosphonate showed a more profound and prolonged hypocalcaemia than did controls. These effects occurred despite adequate PTH levels, unimpaired uptake of ^{45}Ca by duodenal mucosa and normal thyroid calcitonin content. They were taken to imply a direct effect on bone rather than one mediated by hormonal mechanisms. EHDP exacerbated the periparturient decline in total serum calcium in cattle and produced other effects very similar to those described for dichloro-methylene diphosphonate (Yarrington, Capen, Black, Potts, Re and Geho, 1977b).

Treschel, Schenk, Bonjour, Russell and Fleisch (1977) studied a number of diphosphonates. The found that all diphosphonates which inhibited skeletal mineralisation _in vivo_ also inhibited the precipitation of calcium phosphate _in vitro_. However some, which were effective _in vitro_, had no effect on mineralisation. This

suggested that not all diphosphonates altered mineralisation merely by their ability to bind hydroxyapatite.

Although EHDP prevents reabsorption of bone in the short term, its long term effects, more likely to be of clinical relevance, ha received little study. Rosenblum (1974) administered EHDP to rabbits for 28 days and did not find any changes in bone mineral content at doses as high as 10 mg $kg^{-1}day^{-1}$ by subcutaneous injection. Long term use of EHDP in man has been found to have deleterious effects on bone, in particular an increase in the amount of non-mineralised osteoid tissue (Jowsey, Riggs, Kelly, Hoffman and Bordier, 1971). It has been suggested that this effect is only seen with oral doses greater than 20 mg kg^{-1} (Russell and Fleisch, 1975). Experiments in animals have also demonstrated increased non-mineralised osteoid tissue (King, Francis and Michael, 1971; Rosenblum, 1974).

Effects of diphosphonates on experimentally induced calcification defects

Since diphosphonates inhibit bone turnover they are likely to alter the development of pathological defects of calcification. Osteoporosis of rat tibiae and femora can be readily induced by resection of the sciatic nerve or the 2nd-4th lumbar nerve trunks. EHDP or dichloro-methylene diphosphonate reduced the loss of bone ash from immobilised limbs compared with saline controls (Michael, King and Francis 1971; Mulbauer, Russell, Williams and Fleisch, 1971).

Experimentally induced chronic renal failure results in loss of bone mineral and an increase in the number of bone resorptive

sites. EHDP (0.7 mg kg^{-1}) did not prevent these changes (Kaye, 1973). However dichloro-methylene diphosphonate (2 mg kg^{-1}) inhibited bone resorption in renal osteodystrophy (Russell, Termine and Avioli, 1975).

Corticosteroids are known to cause a loss of bone substance. Lindenhayn, Trzenschik, Buhler and Wegner (1982) found that EHDP prevented the bone changes induced by prednisolone treatment in rabbits.

There is good evidence that the neutralisation of excess acid also results in demineralisation. Hydrochloric acid administered to rats and guinea pigs consistently reduced bone carbonate (Irving and Chute, 1932). Administration of ammonium chloride to dogs induced loss of cortical and trabecular bone. The effect was greatest in young dogs and was similar to the effect of a low calcium diet (Jaffe, Bodansky and Chandler, 1932). This work suggested that bone buffers part of an acid load and provided an explanation for an earlier observation; that only 66-86% of an ingested acid load could be accounted for in the urine either as titrable acid or ammonium (Lamb and Evvard, 1919).

Relman, Lennon and Lemann (1961) developed a method for measuring net acid balance in man. It was used to study patients suffering from renal tubular acidosis. These patients excreted significantly less acid in the urine than was produced endogenously (Goodman, Lemann, Lennon and Relman, 1965). It was suggested that the deficit had previously been neutralised by bone resorption. The same effect was observed in normal subjects with ammonium chloride induced acidosis (Lemann, Lennon, Goodman, Litzow and Relman, 1965). This also resulted in negative calcium and phosphorous balances (Lemann, Litzow and Lennon, 1966).

In view of the relation between acidosis, bone and EHDP, Goulding and Broom (1979) studied the effect of EHDP on acid base status in rats. They found, in nephrectomised animals only, that EHDP caused extracellular and intracellular acidosis, and decreased plasma bicarbonate. This is the only study relating to the effect of EHDP on acidosis. However since the studies of Irving et al (1932) and Jaffe et al (1932) several studies of the effect of acidosis on bone mineral have been carried out.

Burnell (1971) used dogs to show that acidosis induced for 5-10 days caused significant reductions in the CO_3/Ca and Na/Ca ratios in bone. During alkalosis these effects were reversed. Studies of the effects of acidosis induced for several months in rodents have also been carried out. Newell and Beauchene (1975) found no change in bone after administration of ammonium chloride to rats. However Barzel and Jowsey (1969), in a similar type of study, observed significant reductions in organic matrix, bone ash, calcium and phosphorous. These changes were independent of dietary calcium, but were reversed by administration of bicarbonate. Dekanić (1979) also found loss of bone ash in rats following induction of acidosis by calcium chloride administration. The effect of EHDP on such changes has not been studied.

CHAPTER II

MATERIALS AND METHODS

1. Preparation of sheep for infusion, and urine and blood collection

Many of the experiments reported in this thesis involved intravenous infusions and the subsequent collection of blood and urine. The day before such procedures female sheep, 20-45 kg, were fitted with catheters under halothane anaesthesia.

For the collection of urine, "Folatex" (Eschmann) balloon catheters (14 to 18 gauge, usually 18 gauge) were used. The 30 ml cuff was only inflated to 10 ml as this was found to be completely effective in retaining the catheter, yet was less likely to induce tenesmus predisposing to leakage of urine. Increased abdominal pressure caused leakage by preventing urine from flowing through the catheter. During the experiment the urine drained via silicone rubber tubing into a plastic measuring cylinder containing approximately 50 mm depth of paraffin oil.

Two venous catheters were fitted in one jugular vein through a small incision. One catheter, a 14 gauge, 120 mm polythene over-the-needle type (Intranule, Vygon), was used for collection of blood. For the intravenous infusion of substances a 2 metre length of PVC tubing (V-3, 2 mm, Bolab Inc) was used. This was inserted through a 14 g needle, in the same vein as the first catheter. The tips of the catheters were approximately 50 mm apart, the infusion

catheter being nearest to the heart. The small incision was closed and the catheters were held in place with monofilament nylon sutures. They were strapped to the sheep's neck with adhesive tape, flushed with 500 i.u. ml^{-1} sodium heparin in saline and sealed until the experiment was to be performed. During an experiment the catheter was flushed with 100 i.u.ml^{-1} sodium heparin after each sample.

For the duration of an experiment the sheep was restrained in a plastic coated steel mesh crate. A mesh partition prevented the sheep from turning and so tangling the catheters. The sheep was prevented from lying down, and thus impeding urine flow, by a surcingle attached to the crate and fitted loosely around the sheep's abdomen. The animal was allowed water ad libitum throughout an experiment, but food was with-held.

Infusions were usually made using a Harvard Apparatus infusion/withdrawal pump. When a higher infusion rate was required, in order to avoid frequent syringe changing, an Ismatec MP-13 peristaltic pump was used.

The experiment was never started until an un-impeded flow of urine, without leakage, was apparent. Sometimes urine failed to flow due to an air lock in the tubing, in that case the injection of 10 ml of sterile water along the catheter usually started urine flow. It was found that at a diuresis greater than 1 ml min^{-1} urine flow was less likely to be troublesome. It was therefore attempted to increase the rate of urine flow. This was done in some experiments by infusing saline solutions at 2.75 ml min^{-1}, with variable results. In other experiments 4 l of water was given by stomach tube 90 minutes before the start of the experiment, This was found to be considerably more effective, but despite these

measures in a number of cases urine flow was still less than 0.5 ml min^{-1}.

Despite the low level of diuresis in many experiments, higher rates of fluid administration were not used since greater levels of saline diuresis increases calcium excretion, decreases proximal tubule reabsorption of both calcium and sodium (Agus, Chiu and Goldberg, 1977), and can slightly reduce plasma calcium (Lins, 1979). Saline infusion always started at least one hour before the start of the experiment and continued until after the post-infusion sampling periods, it is therefore unlikely that any small effects would significantly influence changes produced by the the drug under examination.

2. Materials

All chemicals, unless otherwise stated, were Analytical Reagent grade, and obtained from British Drug Houses Ltd.

3. Collection of blood for measurement of ionized and ultrafiltrable calcium and magnesium

In order to maintain anaerobic conditions prior to preparation of ultrafiltrates, blood was collected in a 10 ml B-D disposable syringe in which the dead space had been filled with lithium heparin (1000 i.u. ml^{-1}). The final blood heparin concentration was 10 i.u. ml^{-1}. When blood was withdrawn from heparinised catheters the first 5 ml was discarded. Blood was drawn into the syringe until the the rear end of the rubber plunger was level with the end of the syringe barrel, the hub was sealed with a plastic cap and

the plastic plunger removed carefully so as not to admit air. Any small bubbles were immediately expelled. The syringe was centrifuged for 20 minutes at 1000 g, the hub being protected by a plastic sleeve in the bottom of a centrifuge bucket of the appropriate diameter. After centrifugation the plasma was withdrawn using a 5ml syringe and 20g needle inserted through the rubber plunger. When determining ionized calcium, blood containing 10 i.u. ml^{-1} lithium heparin was assayed directly. All determinations of ionized calcium and preparation of plasma ultrafiltrates were made within 30 minutes of blood collection. Prior to long term storage, additional heparin was added to prevent clotting of plasma proteins.

4. Total magnesium and calcium determination by atomic absorption spectrophotometry (AAS)

The Perkin Elmer model 103 atomic absorption spectrophotometer was used in conjunction with a Servoscribe 1S potentiometric chart recorder. The absorption of samples was measured at a wavelength of 285.2 nm for calcium, and 422.7 nm for magnesium. Analysis was carried out using a nitrous oxide-acetylene flame. This has a higher flame temperature than the air-acetylene normally used and so eliminates chemical interferences due to phosphate (Carlyle, 1975). The use of a high flame temperature tends to cause ionization of magnesium and calcium in the flame and thus reduces spectral absorption due to atomic calcium and magnesium. This source of error is readily eliminated by adding a relatively high concentration of an easily ionized alkali metal, such as sodium or potassium. Thus potassium chloride (0.2% w/v) (BDH Laboratory

Reagent for AAS) was used for diluting all standards and samples.

Samples were diluted to provide 25-50 micro-mol l^{-1} calcium, and 10-20 micro-mol l^{-1} magnesium. The standards used (BDH Laboratory Reagent, 1 mg ml^{-1} $MgNO_3$; BDH Clinical Reagent 10 mM Ca standard) were diluted to 16.4 micro-mol l^{-1} magnesium and 50 micro-mol l^{-1} calcium. Analyses were performed soon after dilution so as to avoid errors due to absorption of calcium and magnesium by the glass tubes.

To allow time for stabilisation of the burner temperature, at least five minutes elapsed after igniting the flame before measurements were made. It was found that the measured absorbancy was directly proportional to the magnesium concentration over the full range of the machine, extending to 150 micro-mol l^{-1}. For calcium, the relationship between concentration and absorbancy was non-linear above 150 micro-mol l^{-1}. This extent of linearity is significantly greater than the figures quoted by Perkin Elmer for the air-acetylene flame and may reflect superiority of the nitrous oxide-acetylene flame for these determinations. During analysis the machine was zeroed whilst aspirating distilled water, a standard followed by two samples was then aspirated. This procedure was repeated until all the samples had been dealt with.

5. Sample preparation for AAS

5.1 Plasma and ultrafiltrates

Plasma and ultrafiltrates required minimal preparation, all that was normally required was a 1:50 dilution with potassium chloride.

5.2 Urine preparation

Urine required care in handling as some of the urine magnesium and calcium, was in the form of insoluble salts. These were mainly carbonates and bicarbonates and readily dissolved on acidification. Horse urine contained large amounts of acid soluble sediment which was rich in calcium but which also contained some magnesium, as the figures below show.

	Centrifuged urine		Hydrochloric acid treated urine	
Urine (mM)	Ca	Mg	Ca	Mg
Mean (n=15)	1.36	9.60	19.5	11.9

Urine was always thoroughly mixed by shaking before removing a sample for analysis. Initially acidification was carried out using hydrochloric acid, later nitric acid was used. There was no difference in results obtained using either acid. Acidification of sheep urine was carried out by adding nitric acid to give 1% v/v. Horse urine was acidified by adding 5 M nitric acid until liberation of CO_2 ceased. The volume of acid added was corrected for in subsequent calculations. Some non acidified urine was always retained for biochemical assays such as creatinine estimation.

5.3 Faecal preparation and analysis

After weighing the 24 hour collection, the faeces were thoroughly mixed in a large plastic container for five minutes. Portions were then removed for subsequent analysis.

5.3a Faecal dry matter and dry matter calcium and magnesium content

Faecal dry matter was determined by drying a weighed amount of faeces (approximately 200 gm) in an oven at 95°C, until no weight change occurred over a 24 hour period. Portions of faeces (500 gm) were thoroughly mixed and smaller portions of approximately 2 gm were weighed into two pre-weighed porcelain crucibles. These samples were dried for 48 hours at 95°C and their weights recorded. They were ashed in a muffle furnace at 300°C for 12 hours followed by 500°C for a further 24 hours. The crucible was then placed on a gauze several inches above a low bunsen flame. Aliquots (1.5 ml) of 0.5 M hydrochloric (BDH, Aristar Reagent) acid were added and transferred, when hot, to a 10 ml volumetric flask. This process was continued until the flask was almost full. Care was taken to ensure that all solid matter was transferred, thoroughly washing down the sides of the crucible. When cool, the flasks were topped up with hydrochloric acid and sealed. Over the next two to three days they were periodically mixed by inversion, the supernatants were then saved for analysis by AAS. Faecal calcium and magnesium were calculated on the basis of the dry weight of faeces in the crucibles.

5.3b Faecal fluid total and ultrafiltrable calcium and magnesium content

Faecal fluid was extracted by compressing the faeces in a thick walled plastic cylinder with a piston driven by a screw jack. The resultant fluid percolated out through holes in the base of the cylinder and was collected in a plastic beaker below. The extract was centrifuged for 1 hour at 1000 g to remove coarse debris and was then assayed directly by AAS after appropriate dilution. Ultrafiltrates of the faecal fluid were also prepared, as described over the page, and analysed by AAS. The 24 hour excretions of calcium and magnesium in faecal water fractions were calculated, for example for calcium:

$$Ca_{24hours} = \text{faecal weight} \times (1 - (\text{dry matter \%} \div 100)) \times [Ca]$$

5.4 Mouse femur preparation

Within one hour of death the left femur of each mouse was carefully dissected out. The bones were then dried at 105°C for six hours, allowing easier removal of excess soft tissue, before drying for a further 18 hours. They were allowed to cool in a desiccator and weighed. Each set of dried bones was defatted by refluxing, for 12 hours, in 100 ml methanol/trichloro-ethylene (50% v/v of each) which was replaced with fresh solvent at four hourly intervals. After draining, the excess solvent was allowed a few minutes to evaporate before the bones were dried in an oven at 105°C for six hours. They were then cooled in a desiccator, weighed in tared and labelled porcelain crucibles, and the fat-free weights recorded. Ashing was carried out in a muffle furnace at 300°C for 3 hours

followed by 600°C for 24 hours. This temperature, higher than that used in 5.3b , was not associated with losses of sodium, potassium, calcium or magnesium from samples of the chloride salts. The ash was allowed to cool and was then extracted hydrochloric acid as described in 5.3a.

5.5 Preparation of ultrafiltrates

Visking tubing 5.2 cm circumference (Scientific Instrument Centre Ltd) was cut into lengths 3 metres long which were then connected to a mains cold water supply and rinsed through with soft tap water for about three hours. This process served to remove gross contamination. The excess water was then squeezed out and the tubing was placed in a beaker of distilled water which was changed every day for approximately fourteen days. Both ends of the tubing were connected to a manifold to dry the tubing by inflating with O_2/CO_2 from a gas cylinder. After two hours the tubing could be cut into lengths 10 cm long and stored ready for use.

Prepared visking tubing was folded longitudinally and then bent double and put in a polystyrene tube (1.5 by 9.0 cm) with both open ends protruding. The polystyrene tube was gassed with 95% O_2 5% CO_2 delivered to the bottom of the container via fine tubing. The sac was then filled with 1.5-2.0 ml of plasma from a 5 ml syringe. The sac was protected from perforation by a small piece of PVC tubing slipped over the end of the needle. The tube was briefly gassed again before sealing with a plastic cap, so that the ends of the visking tubing were trapped outside the polystyrene tube. Parafilm (Gallenkamp) was then wound in a narrow strip around the end of the tube to assist in forming a gas tight seal.

All samples were ultrafiltered in duplicate.

The tubes were placed in a MSE Mistral 2L centrifuge whose temperature had been raised to 37°C by running it at 2300 rpm for approximately one hour. A swing out rotor was used since the angle rotor drastically reduced the yield of ultrafiltrate. The tubes were then spun at 900 g for two hours, yielding 0.3-0.4 ml of ultrafiltrate. The visking tubing and its contents were then removed and calcium and magnesium assayed in the ultrafiltrate.

6. Measurement of ionized calcium using the Nova-2 Ca^{++} Analyser

The Nova-2 calcium analyser (American Hospital Supply UK Ltd), consists of a microprocessor controlled flow-through electrode system thermostatted at 37°C. The machine is highly automated: standardisation, checks for drift, instability of electrode emf, reduction in electrode response and the presence of air bubbles are automatically made and indicated as detected. The machine performs a two point calibration using 1.0 and 2.0 mM calcium standards (containing sodium chloride to provide an approximately physiological ionic strength) every two hours, or as required. It also analyses a 1.0 mM standard after each sample.

During _in vivo_ experiments calcium concentration was always measured on freshly drawn whole blood. The presence of blood cells does not affect the electrode response to calcium ions. Blood or plasma was aspirated directly from the syringe. The system was checked by analysing 0.5 and 1.5 mM standards (American Hospital Supply UK Ltd) daily. Blockage of the reference electrode was a recurring problem, this was usually readily overcome by flushing the electrode with distilled water.

7. Measurement of ionized divalent cations using the method of Heaton (1967)

7.1 Preparation of ion exchange strips

The cation exchange strips used in the original study were no longer available so a simple method for producing them in the laboratory was developed. Visking tubing, 5.2 cms circumference was split longitudinally along its flattened edges. These lengths were then carefully cut into 4 cms strips (the coefficient of variation of the weight of 50 strips was 1.4%). Batches of strips were soaked in five changes of distilled water, changed at 24 hour intervals. Excess water was then drained and the strips transferred to a magnetically stirred solution of congo red (May and Baker Ltd, 0.5% w/v, in 20% v/v industrial alcohol) at 60°C. After four hours the strips were removed and rinsed in distilled water which was changed daily until only a faint tinge of pink remained in the discarded washings. The final rinses were made in 150 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$ (diluted from 1 M $CaCl_2$, BDH Ltd Volumetric solution) and 0.5 mM $MgCl_2$ (diluted from BDH Ltd, Clinical Reagent 1 M Mg).

7.2 Estimation of ionized calcium and magnesium in plasma

Samples were handled anaerobically (as previously described). Plasma (3 ml) was then transferred to a polystyrene tube which had been gassed with 95% CO_2, 5% O_2. Using forceps, an ion exchange strip was vigorously shaken to remove excess moisture and placed in the plasma. The tube was then briefly gassed again, sealed with a plastic cap and placed in a Gallenkamp shaking water bath at 37°C.

After 10 minutes the strip was removed from the sample. It was

briefly rinsed in a jet of double distilled water and shaken in order to remove excess plasma. Elution was carried out by placing the strip in a 6 ml glass test tube containing 5 ml of potassium chloride (0.2% w/v, Aristar grade, BDH Ltd) for 30 minutes. Adequate mixing was achieved by attaching the tubes to a metal disc which rotated at 8 rpm in a vertical plane, the tubes long axis was aligned on the disc radially, as a spoke of a wheel, so that with each revolution it was inverted once.

After elution the solution was analysed, without further dilution, by AAS. The measured eluate calcium and magnesium concentrations were then compared with calibration curves prepared using 5 ml samples of standard solutions. These solutions were of the basic composition: 150 mM NaCl, 4 mM KCl and 6 mM tris-HCl buffer (pH 7.40); the concentration of magnesium was varied in six equal steps, from 0.2 to 1.2 mM; calcium was similarly varied, from 0.2 to 2.2 mM.

8. Estimation of ionized magnesium and calcium in vitro

Studies of the effects of complexing agents on ionized magnesium or calcium in aqueous solutions were performed using either the Orion Research 93-20-01 calcium ion electrode or the 93-32-01 divalent cation electrode in conjunction with the 90-01 single junction reference electrode. The divalent cation electrode suffered severe interference in the presence of 0.16 M sodium and lesser interference from potassium. When using this electrode experiments were therefore carried out in solutions containing low sodium and potassium concentrations.

The same concentration of tris-HCl buffer was used in standard

and test solutions, this was necessary since the divalent electrode is sensitive to tris. Over the concentration range 10^{-2} to 10^{-4} M, tris buffer (pH 7.34) was found to produce an electrode response of 15 mV per 10 fold change in concentration (Kent, Bunce, Bailey and Aikens, 1974).

It was always attempted to bracket each sample with standards of higher and lower magnesium or calcium concentration. At very low concentrations this was not possible. In the EHDP binding studies (Chapter VI) standards of 0.04, 0.07, 0.10, and 0.70 mM were used. The electrode emf was measured using either an EIL 33B-2 vibron electrometer, or a Fluke digital mV meter (input impedances of 10^{15} ohms and 10^{6} ohms respectively). At a given calcium concentration the emf recorded by the Fluke mV meter was slightly lower than that of the electrometer presumably due to its relatively low resistance, however this did not affect the measured ionic activities. All readings were taken at room temperature.

The electrodes displayed a variable degree of drift during use. After placing the electrode in a solution the electrode emf stabilised in approximately 60 seconds, thereafter the emf continued to change at a slower rate. The emf was not recorded until reasonably steady values had been reached.

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9. Sodium and potassium determination

These ions were determined by means of a Corning EEL Clinical Flame Photometer. Plasma was diluted 1:200 with distilled water, urine was diluted as necessary. Standards containing 200 mM sodium and 10 or 100 mM potassium were prepared and diluted 1:200 for use. The propane/air mixture was adjusted for maximum light emission from an aspirated standard solution and ten minutes allowed for stabilisation before any samples were analysed.

10. Measurement of urinary pH, blood gases and pH

Heparinised blood was collected anaerobically and analyses made as soon as possible. Blood pCO_2 and pH were measured using the Corning model 165 pH/blood-gas analyser. This machine uses the Henderson-Hasselbalch equation to calculate values for bicarbonate. The values for base excess and total CO_2 are calculated from constants used in man, which are probably not accurate in the sheep due to the low 2,3 diphosphoglyceric acid concentration in sheep blood. It was therefore considered preferable to use the nomogram of Gattinoni and Samaja (1979).

Urine was collected under oil to prevent loss of CO_2. When urine pH was required the measurement was made as soon as possible after collection of the sample, with a Pye Unicam model 290 pH meter and a Russell glass pH electrode.

11. ^{14}Carbon scintillation counting

^{14}C-EDTA was measured in plasma and urine collected from sheep during intravenous infusions of EDTA. Counting was carried out in a Corumatic-200 liquid scintillation spectrometer (ICN Tracerlab) after mixing with Aquasol 2 (New England Nuclear) in glass scintillation vials. Counting was performed for 500,000 counts, or for at least fifty minutes. The method posed few problems. The main one was the reduction in counting efficiency, or quenching effect, due to the aqueous nature of samples such as plasma and urine (figure 2.1).

To minimise quenching errors standardisation was carried out, for each experiment, by counting four or five plasma samples before and after the addition of aliquots of the infusion solutions containing approximately 20 nano-Ci of the tracer ^{14}C-EDTA. The same procedure was also used in urine when the level of diuresis changed little. In some experiments the diuresis was very low, with a resultant increase in the concentration of the urochrome pigments present. This caused a significant increase in the quenching produced by urine in addition to the quenching effect due to the aqueous phase. This, in itself, was not a problem since the concentration of EDTA in antidiuretic urine was also high, so allowing the use of higher dilutions. However when the diuresis varied greatly during any one experiment it was necessary to use internal standards for every urine sample in order to overcome this variability.

When EDTA infusions into sheep were to be made, 8 micro-Ci of ^{14}C-EDTA (specific activity 108 Ci mol^{-1}; Radiochemical Centre, Amersham) was added to the required amount of cold EDTA. This was

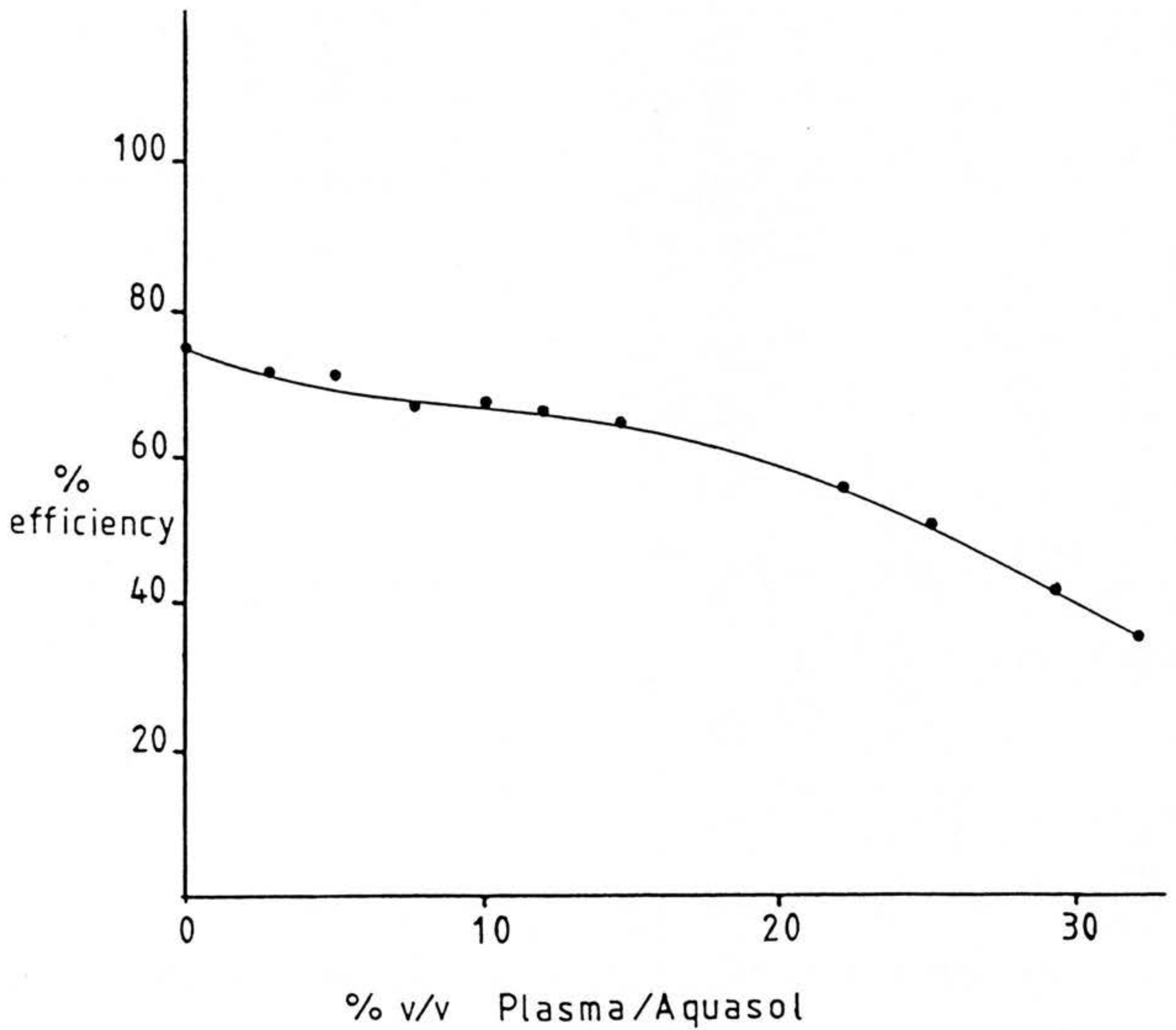


Figure 2.1 The quenching effect of plasma on the efficiency of scintillation counting of ^{14}C-glucose counting in Aquasol-2.

then used for infusion after retaining some for internal standardisation of urine and plasma. For measurement of ^{14}C in plasma, 3 ml was mixed with 10 ml of aquasol. With urine this was reduced, sometimes only 0.05 ml was required, depending on the level of diuresis and the resultant EDTA concentration. However the dilution was kept constant for any single infusion.

12. Creatinine estimation

Measurement of creatinine in urine and plasma was based on the method of Owen, Iggo, Scandrett and Stewart (1954), as described by Varley (1980). The method described below incorporates a number of minor modifications.

12.1 Reagents:

(1) 0.15 M sodium tungstate.

(2) 0.33 M sulphuric acid.

(3) 10% suspension of Lloyd's reagent (BDH Fullers earth for adsorption purposes).

(4) Saturated oxalic acid solution.

(5) 2.5 M sodium hydroxide.

(6) Saturated picric acid.

(7) Stock creatinine zinc chloride 10 mM (BDH Laboratory Reagent) was stored at $4^{o}C$ and prepared fresh every 4 weeks.

Prepared fresh for use:

(1) Standard creatinine (stock was diluted 1:250 or 1:500).

(2) Alkaline picrate (27.5 ml saturated picric acid plus 5.5 ml sodium hydroxide, diluted to 100 ml with distilled water).

12.2 Method:

Plasma was deproteinated by mixing 0.75 ml each of plasma, distilled water, tungstate and sulphuric acid reagents followed by shaking and centrifugation.

Two ml of deproteinised supernatant, 0.2 ml of oxalic acid and 0.4 ml of Lloyds reagent (re-suspended immediately before withdrawing an aliquot), were placed in a round bottomed test tube which was then stoppered and shaken to suspend the solid. The tubes were mixed continuously by attaching the tubes to a metal disc which rotated in a vertical plane at 8 rpm, the long axis of a tube was aligned radially as the spoke of a wheel, so that with each revolution it was inverted once.

At the end of this period the tubes were centrifuged. The supernatants were then decanted and the tubes inverted onto filter paper to drain.

After five minutes 2 ml of alkaline picrate was added and the Lloyds reagent resuspended by mixing on a vortex mixer (round bottomed tubes were preferable to conical centrifuge tubes as the Lloyds reagent re-suspended more readily). They were then restoppered and mixed for a further 10 minutes on the rotating disc.

Finally the tubes were centrifuged at 1000 g at room temperature. The absorbance of the supernatant was then measured at 520 nm in a glass micro-cell with a 1 cm light path, using a Cecil Instruments CE 303 grating spectrophotometer.

Standards and distilled water blanks were also analysed in an identical way. Urine did not require deproteinisation, it was usually diluted 1:400 before analysis.

Duplicate estimations of urinary creatinine gave a mean coefficient of variation of 2.16% (n=11). In plasma, probably as a result of the very low creatinine concentration, the coefficient of variation was 3.78% (n=30). Recovery of creatinine added to urine was 98.3 $\pm$ 1.2% (n=5). The accuracy of the method was more than adequate for the determination of clearances and was greater than that of the inulin assay which is usually considered to be the more accurate measurement.

13. Inulin estimation

Inulin was measured by the method of Davidson and Sackner (1963). The method is a modification of the anthrone method (Young and Raisz, 1952), in which the anthrone concentration is reduced to 0.08% and the incubation temperature reduced to 38°C. These modifications were shown by Davidson and Sackner (1963) greatly to reduce the interference produced by glucose.

13.1 Reagents:

(1) 1.0 M trichloroacetic acid.

(2) Anthrone reagent; 100 ml sulphuric acid was slowly added to 26 ml distilled water and allowed to cool, in this was then dissolved 0.1 gm anthrone (BDH Ltd Laboratory Reagent; recrystallised twice from hot glacial acetic acid). This solution was prepared fresh every 48 hours, and stored at 4°C in a dark bottle.

(3) Stock inulin standard 1 mg ml^{-1} (Koch-Light Laboratories Ltd, Inulin Puriss.). The same inulin was used for standardisation and infusions.

13.2 Method:

Plasma (0.2 ml), 1.0 ml water and 0.8 ml 1.0 M trichloroacetic acid were placed in a test tube, shaken thoroughly and centrifuged at 1000 g for 20 minutes. The supernatant was saved for analysis.

Aliquots (3 ml) of the anthrone reagent were cooled in test tubes in iced water. Then 0.5 ml of each supernatant was added to the tubes which were stoppered, thoroughly mixed and returned to the iced water.

The tubes were incubated in a water bath at 38°C for 50 minutes, then rapidly cooled in iced water. The absorbance of samples, appropriately diluted standards and blanks were read at 620 nm in glass cells with a 1 cm light path using a Cecil Instruments CE 303 grating spectrophotometer.

Urine was analysed after appropriate dilution, without deproteinisation. Urine and plasma blanks were made from the urine and plasma collected before the start of the inulin infusion.

The coefficient of variation of the method was 3.19% (n=29) in plasma and 4.37% (n=21) in urine. The plasma inulin concentration, when infusions were made as described in the Appendix, was 20-30 mg $100ml^{-1}$. The absorbance of the blanks was negligible.

14. Calculation of renal clearances

The renal clearance of a substance, for example creatinine, (where Vol_{urine} is in ml $hour^{-1}$), was calculated as follows:

$$Creatinine_{Clearance} = \frac{Vol_{urine} \times [Creatinine]_{urine}}{[Creatinine]_{plasma} \times 60} \text{ ml min}^{-1}$$

For the calculation of inulin clearance, plasma inulin concentration was determined in blood samples collected at the beginning and end of each hourly period of urine collection and the mean calculated. Since plasma creatinine concentration varied very little during the four to eight hours of an infusion, creatinine clearance was calculated from the mean value of plasma creatinine determined during the whole period of the infusion. During the experiments in the horse, in which 24 hour values for creatinine clearance were determined, clearance was calculated from the daily plasma creatinine concentration.

15. Statistical analysis

Results were compared with control values using a paired t-test for paired data. The t-test for the un-paired case was used to compare un-paired groups of data. In the experiments described in Chapter VIII a large number of t-tests would have been required to evaluate the data. In order to reduce the risk of producing false significance associated with such multiple comparisons the data was first analysed using analysis of variance. Where this revealed significant differences, the individual groups were then compared with the unpaired t-test to reveal where such differences lay.

Correlation coefficients, regression lines and their confidence limits were calculated by the method of least squares. All data in the tables and figures are shown as mean $\pm$ standard error of the mean.

CHAPTER III

EVALUATION OF METHODS FOR ESTIMATING FRACTIONS OF PLASMA CALCIUM AND MAGNESIUM

Summary

1. Low capacity ion exchange strips were examined as a potential method of measuring ionized calcium and magnesium. Ion exchange strips were equilibrated with test solutions and the adsorbed cations eluted into potassium chloride solution. The concentration of calcium and magnesium in eluates was proportional to their concentrations in standard solutions and inversely proportional to the concentration of interfering cations, though the latter effect was small. Unfortunately complexed magnesium was as readily adsorbed by the ion exchange strips as the ionized fraction. It was concluded that the method did not specifically measure ionized magnesium.

2. The Nova-2 calcium ion analyser was examined. The linearity of the response of electrode emf to changes in log calcium concentration, reproducibility and response to added calcium, EDTA or heparin in blood was studied. Provided heparin concentration was low and carefully controlled it was found to be a satisfactory method of measuring ionized calcium.

3. The suitability of a method using visking tubing for the preparation of plasma ultrafiltrates was investigated. The method was influenced by Donnan shifts and temperature. Small increases in pH occurred during ultrafiltration which would be expected to slightly depress ultrafiltrable calcium and magnesium concentrations. This effect was less than the inherent variability of the method.

MATERIALS AND METHODS

These have been described in Chapter II.

RESULTS AND DISCUSSION

I

Low capacity ion exchange strips for measuring ionized calcium and magnesium

1. Calibration of the method

Calibration curves were prepared as described in Chapter II, section 7.2. As expected, the magnesium concentration in the eluate was directly proportional to the magnesium concentration in the standard solution (figure 3.1). The correlation coefficient was highly significant ($r=0.999$, $P<0.001$). Calcium also showed the same relationship ($r=0.996$, $P<0.001$).

2. The effect of equilibration of ion exchange strips on the composition of plasma

Total calcium and magnesium concentrations were measured by AAS in 10 plasma samples before and after ion exchange strips were equilibrated in them. Ion exchange strips did not cause measurable changes in either calcium or magnesium concentration: the calcium concentration was 2.40 ± 0.05 mM pre-equilibration and 2.41 ± 0.05 mM post-equilibration; the magnesium concentration was 0.801 ± 0.03 mM pre-equilibration and 0.798 ± 0.04 mM post-equilibration.

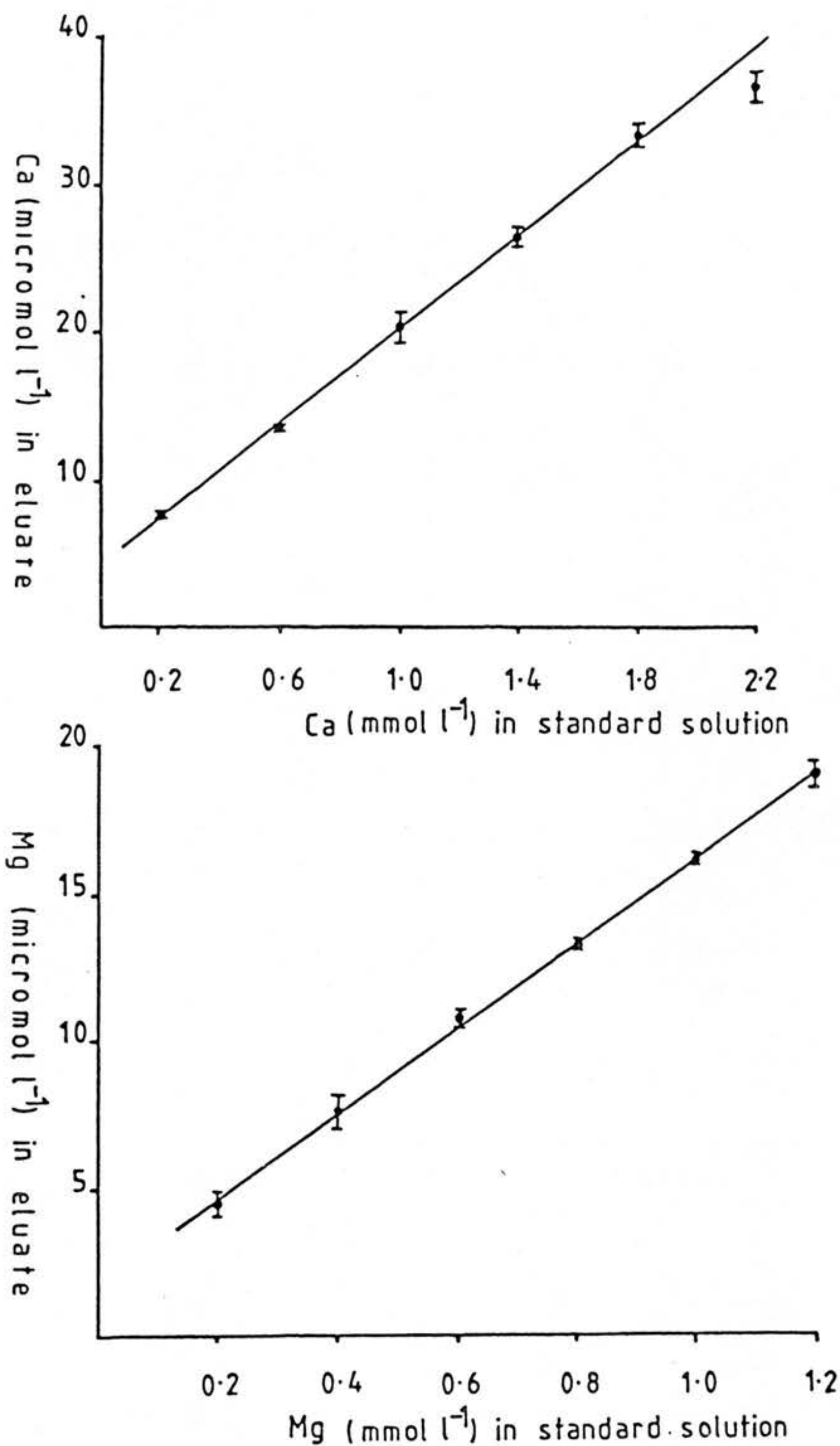


Figure 3.1 The effect of changes in the concentration of calcium or magnesium in standard solutions on the concentration of calcium or magnesium in eluates from ion exchange strips. Each point is a mean and standard error of four observations.

3. The effect of alterations in the composition of the standard solution on calcium and magnesium concentrations in the eluate

The interference produced by alterations in the constituents of the standard was investigated by independently varying the constituents over a range of concentrations. The concentration ranges used are shown in table 3.1. The basic composition of the standard was: 150 mM NaCl, 4 mM KCl, 6 mM tris-HCl (pH 7.40), 1.0 mM $CaCl_2$ and 0.6 mM $MgCl_2$.

The concentration of magnesium in the eluate decreased as the concentration of tris ($P<0.02$), calcium ($P<0.02$) or sodium ($P<0.001$) in the standard solution increased (figure 3.2). Figure 3.3 shows that eluted calcium was similarly affected by sodium ($P<0.001$) and magnesium ($P<0.05$), though the effect of tris was not significant. Potassium had no significant effect on either ion. The calculated gradients of the regressions in figures 3.2 and 3.3 are shown in table 3.1.

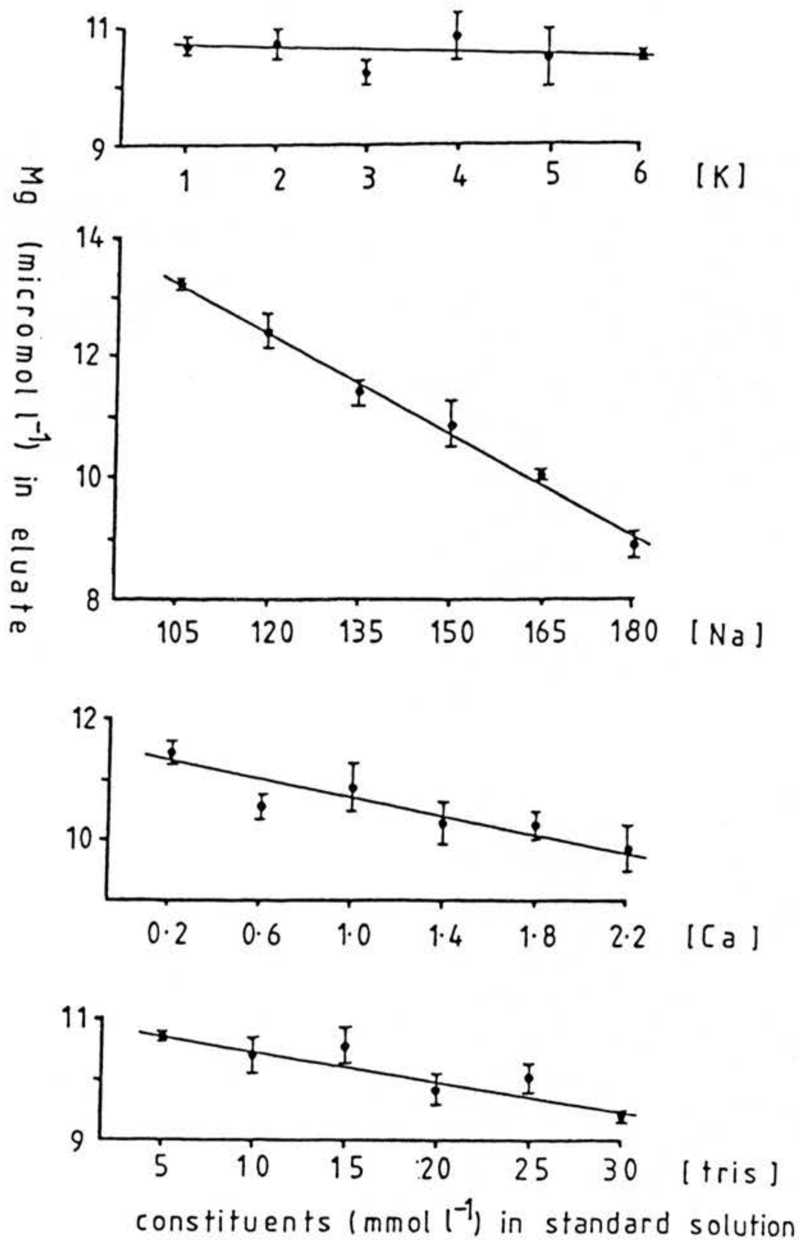


Figure 3.2 The effect of changes in the concentration of the constituents of the standard solution on the concentration of magnesium in eluates from ion exchange strips. Each point is a mean and standard error of four observations.

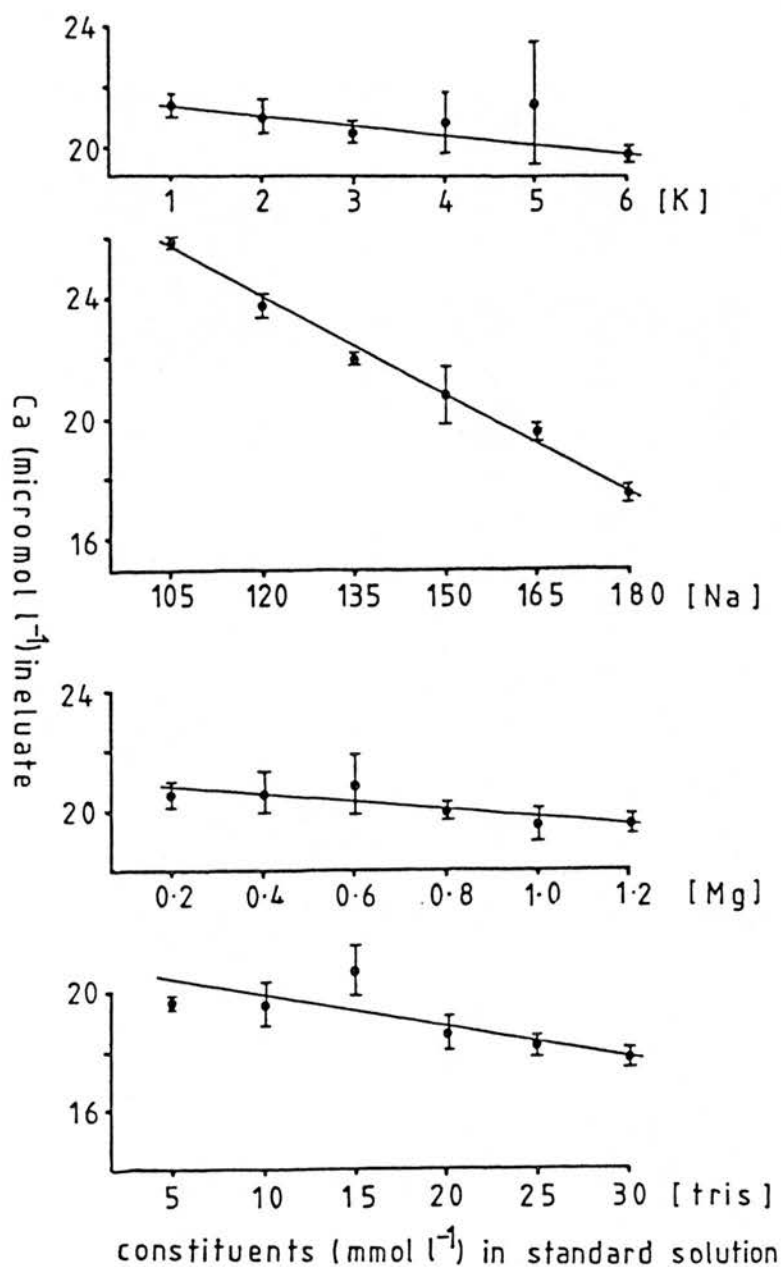


Figure 3.3 The effect of changes in the concentration of the constituents of the standard solution on the concentration of calcium in eluates from ion exchange strips. Each point is a mean and standard error of four observations.

Table 3.1

The gradients of the regressions of eluate magnesium or calcium concentration on the independently varied concentrations of each constituent of a standard solution

Varying constituent	Range studied (mM)	[magnesium]: [constituent] grad. (x 10^{-3})	P cf 0.0	[calcium]: [constituent] grad. (x 10^{-3})	P cf 0.0
Potassium	(0-6)	-0.051 ± 0.055	>0.1	-0.191 ± 0.138	<0.1
Sodium	(105-180)	-0.056 ± 0.002	<0.001	-0.105 ± 0.005	<0.001
Tris	(5-30)	-0.049 ± 0.011	<0.02	-0.089 ± 0.036	<0.1
Calcium	(0.2-2.0)	-0.668 ± 0.158	<0.02	+14.7 ± 0.4	<0.0001
Magnesium	(0.2-1.2)	+14.4 ± 0.1	<0.0001	-1.130 ± 0.367	<0.05

The values are gradients ± standard errors calculated from 24 observations. Each value was calculated from the regressions of [adsorbed cation] vs [interfering cation] shown in figures 3.1,3.2 and 3.3, and represents the calculated gradient for each line. The nominal composition of the standard was 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 4 mM KCl, 150 mM NaCl and 6 mM tris-HCl (pH 7.40).

4. The effect of complexing agents on eluted magnesium

Standard solutions contained: 150 mM NaCl, 4 mM KCl, 6 mM tris-HCl (pH 7.40), 0.6 mM $MgCl_2$ and either: EDTA (0 to 6.0 mM); trisodium citrate (0 to 3.0 mM); or EHDP (0 to 6.0 mM). These solutions were equilibrated with ion exchange strips and the adsorbed cations eluted into potassium chloride solution. Eluate magnesium concentrations were assayed by AAS. Standard solutions containing varying magnesium concentrations were also assayed enabling values for ionized magnesium to be obtained.

The correlation between measured ionized magnesium and the concentration of complexing agent was not significant for either citrate (r=0.69, n=5), or EDTA (r=0.27, n=7). Figure 3.4 shows very clearly the deviation of measured ionized magnesium concentration from calculated values in the presence of citrate or EDTA.

In saline solution the correlation between EHDP concentration and measured ionized magnesium was significant (r=0.92, n=5, $P<0.05$; table 3.2), however the reduction in measured ionized magnesium was extremely small (2.4% per mM change in EHDP). Finally, ion exchange strips were equilibrated with 5 ml samples of plasma containing 0.6 ml aliquots of saline or EHDP solutions (0 to 60 mM). Plasma ionized magnesium was not significantly altered by EHDP (r=0.37, table 3.2).

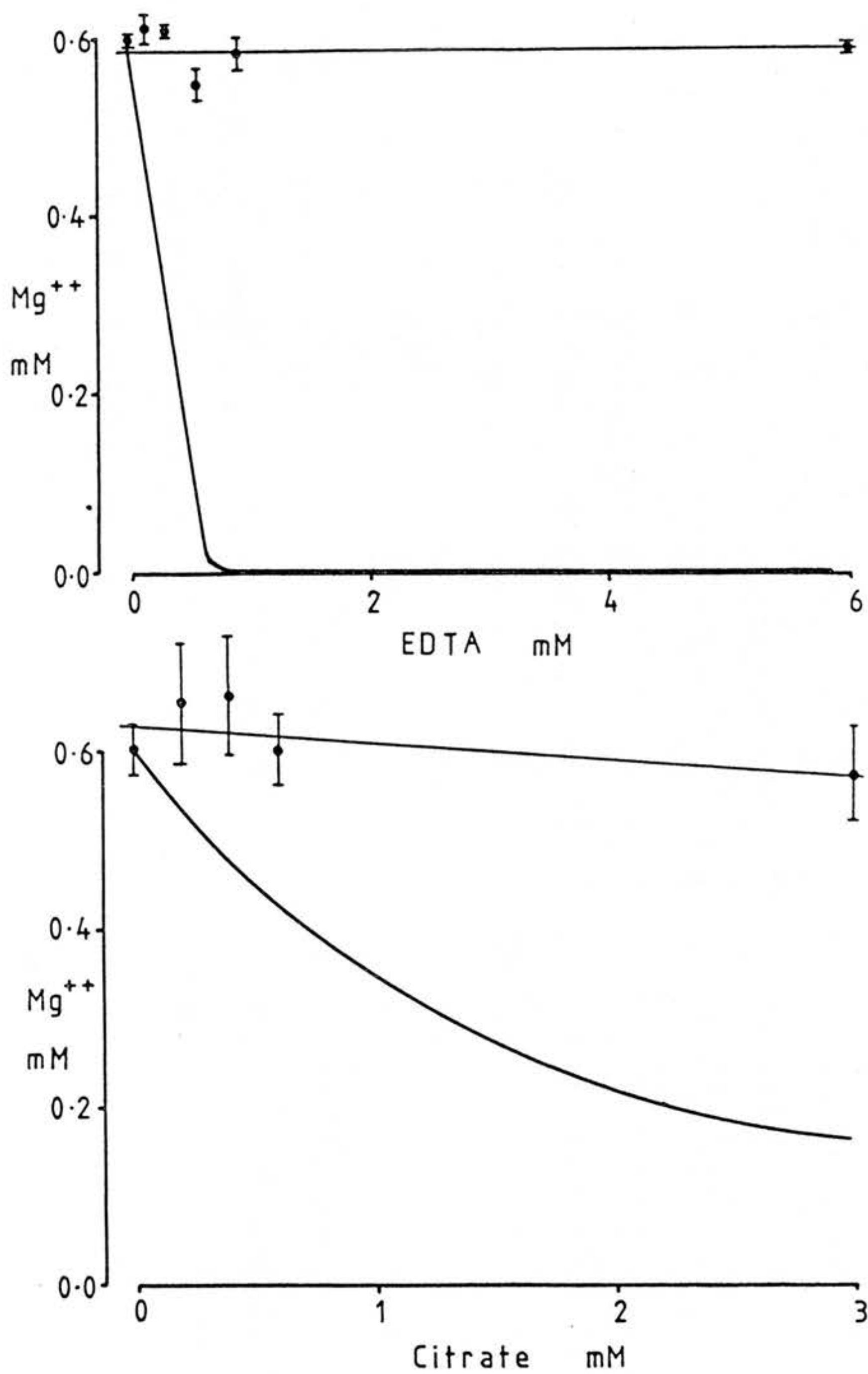


Figure 3.4 A comparison of measured and theoretically derived values for ionized magnesium concentration in aqueous solutions containing EDTA or citrate. Theoretical values (heavy lines) were calculated from the binding constants at pH 7.40: Magnesium citrate, 10^{-3} M; Magnesium EDTA, 10^{-6} M. Each point is a mean and standard error of four observations.

Table 3.2

Measurement of ionized magnesium, using the method of Heaton (1967), in plasma or saline solutions containing EHDP

EHDP saline mM	Ionized magnesium mM	
0.0	0.60 ± 0.02	(4)
1.0	0.59 ± 0.03	(4)
2.0	0.57 ± 0.04	(4)
4.0	0.54 ± 0.04	(4)
6.0	0.50 ± 0.04	(4)

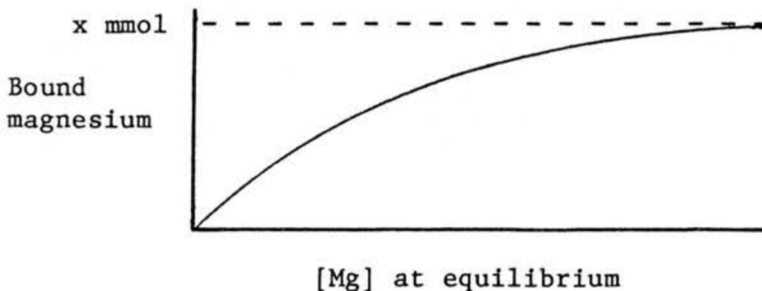
EHDP plasma mM		
0.0	0.60 + 0.03	(4)
0.6	0.56 + 0.02	(4)
2.3	0.57 + 0.03	(4)
4.4	0.60 + 0.04	(4)
6.4	0.58 + 0.03	(4)

Figures are means ± standard errors (number of observations). The correlation between EHDP and ionized magnesium was calculated for saline and plasma ($r = 0.92$, $P<0.05$ and $r = 0.37$, $P>0.1$ respectively).

Discussion

Low capacity ion exchange strips: theoretical considerations

In order to appreciate the implications of some of the findings reported in this work it is first necessary to discuss some of the theory and assumptions involved in the method. Low capacity ion exchange strips are assumed to have a fixed number of cationic receptor sites. In a solution containing a vast excess of a cation such as sodium, all receptors will be occupied by that ion. If a low concentration of a second ion having a similar affinity for the receptors is added to the solution it will displace some of the sodium. As the concentration of the second ion increases, the amount adsorbed by the ion exchange strips will also increase. Not until its concentration is many times that of sodium will effectively all the cationic receptors be occupied by the second ion. If that second ion is magnesium then the relationship between the ionized concentration of magnesium at equilibrium and the total bound magnesium can be described graphically. For an ion exchange strip capable of binding x mmol of cations the following relationship is hypothesised:



Reducing sodium concentration will increase bound magnesium and so

shift the curve to the left. Increasing sodium concentration will decrease bound magnesium and so shift the curve to the right. Similarly, an increase in the affinity of the receptors for magnesium or a decrease in the affinity for sodium will shift the curve to the left, the converse is also true. As the affinity for magnesium increases the curve will approach closer and closer to the x axis. At infinitely high magnesium affinity bound magnesium will no longer be related to magnesium concentration at equilibrium but to the total magnesium present in the solution or the total number of receptor sites, which ever is the smaller.

In plasma, because of the relative affinities of ion exchange strips for sodium and magnesium and because the concentration of sodium is much higher than magnesium, bound magnesium constitutes only a very small fraction of the total bound cations. The result is that the portion of the bound versus ionized magnesium curve very close to the origin is the only part of the curve which is of relevance. Experiment has shown that over this portion of the curve the relationship is, to all intents and purposes, linear (Heaton, 1967).

In order to accurately measure ionized magnesium it is also important that the total capacity of the ion exchange strips is low so that the concentration of the various ions in the sample is not substantially altered. This would affect ionic strength and magnesium-ligand equilibria, both changes which would affect ionized magnesium.

It seems that several criteria might be suggested which may indicate the suitabilty of any low capacity ion exchange method for measuring ionized cations.

(1) It is axiomatic that the cation exchange strips must only

adsorb ionized species and that the the adsorbed cations must be available for elution.

(2) The ratio of measured to interfering cation (eg. magnesium/sodium) in the sample must be small.

(3) The affinity of the cation exchange strips for the measured cations must fall within limits.

(4) The total capacity of the ion exchange strips must be low, so that in plasma samples, when all the cationic sites are occupied, minimal changes in composition occurs.

Criteria 2 and 3 are difficult to quantify, suffice it to say that if experiment demonstrates a linear relationship between bound and ionized magnesium over an appropriate concentration range, it is reasonable to assume that these criteria are being satisfactorily met. Although magnesium was the cation considered in this discussion, the method should be equally applicable to any cation which fits these criteria. In the studies described some of these criteria were investigated.

<u>Comparison of the method used with that of Heaton (1967)</u>

Since the procedure used by Heaton (1967) was slightly modified for use in this study it is pertinent consider methodological differences that might invalidate the proposed method. These modifications were introduced to improve the method. The main modifications were: the use of magnesium and calcium containing solutions for storing ion exchange strips prior to use; the use of 37°C instead of room temperature for equilibration of the ion exchange strips; the use of tris instead of bicarbonate buffer in standard solutions; elution in 0.2% potassium chloride instead of

5% sodium chloride; the source of the ion exchange material.

By equilibrating ion exchange strips with 0.6 mM magnesium and 1.0 mM calcium prior to use, the subsequent change in ionized calcium and magnesium in the sample under investigation was minimised since the ion exchange strips already contained approximately the amount of calcium and magnesium that would be adsorbed from the solution.

The use of 37°C can only improve the method since gas partial pressures and thus plasma pH will be maintained at physiological levels. As described in the introduction these factors are important in determining ionized calcium and magnesium.

Tris was the preferred buffer since bicarbonate is known to complex magnesium and requires gassing with CO_2/O_2 during use. Calibration curves prepared using bicarbonate and tris buffers revealed no differences.

Heaton (1967) used 5% sodium chloride for elution, presumably in the hope that a relatively high concentration of sodium would displace the adsorbed cations most effectively. However the calcium and magnesium concentration in eluates was not significantly different after elution into either 0.2% potassium chloride or 5% sodium chloride. Potassium chloride (0.2%) was preferred as it is a more suitable ionization buffer for AAS, and as high concentrations of salts can cause inaccuracy during analysis due to solids accumulating on the burner.

The modifications described so far did not adversely affect the method. The properties of the ion exchange strips are probably the most likely source of discrepancies between the published results of Heaton (1967) and those of the present study. The magnesium adsorbing capacity of the strips calculated from calibration curves

was greater in the present study than in the original work ($P<0.05$). The difference was not observed in batches of ion exchange strips prepared using a lower concentration of congo red; it thus seemed to be due to a higher content of the adsorbing dye and of no significance.

In the original work it was shown that a 37 mM decrease in the sodium concentration of the standard solution increased the magnesium concentration in the eluate by 120%. In the present study calculation from the data in figure 3.2 gives the same result: 120 $\pm$ 1.6% (mean $\pm$ SEM). However there appear to be quantitative differences in the relative susceptibility to interference from potassium and calcium of the original method and that described here, since Heaton's data fall outside 95% confidence limits calculated from the data presented in table 3.1. Since Heaton made few observations, the apparent differences may merely be a result of experimental variation.

Validation of the method

It was not possible to detect any alteration in plasma total calcium or magnesium before or after equilibration of ion exchange strips. This is not surprising since the capacity of the strips was very low and prior to use they were equilibrated with solutions containing 1.0 mM calcium and 0.6 mM magnesium. The method clearly does not alter plasma calcium or magnesium concentrations sufficiently to have a significant effect on protein and ligand binding equilibria.

In this study the concentration of magnesium in the eluate was directly proportional to the concentration of magnesium in the

standard, calcium showed the same relationship. This confirmed the expected linear relationship between eluted cations and cation concentration. Since the amount of magnesium or calcium adsorbed by the ion exchange strips was also inversely proportional to the concentrations of interfering cations present in the standard solution, competition for the binding sites must have occurred. The change in eluate calcium and magnesium induced by alterations in interfering cations was less than 1% of that resulting from equimolar alterations in measured cations. Clearly the method is very sensitive to changes in the measured cation and relatively insensitive to changes in interfering ions.

Ions which have a large effect on eluted calcium or magnesium must displace divalent cations from the cationic binding sites and so be bound more strongly by these sites than ions which have little effect. Thus the gradients of the interference regressions shown in table 3.1 indicate the relative affinities of the ion exchange strips for different cations. The gradients of the regressions were similar for all monovalent cations which seem, therefore, to have similar affinities for the cationic receptor sites. Changes in calcium concentration in the standard had a ten-fold greater effect on eluted magnesium concentration than did equimolar changes in monovalent cations. Similarly, the effect of magnesium on eluted calcium was ten times that of the monovalent cations. Thus it seems that the ion exchange strips have a ten-fold greater affinity for divalent than for monovalent ions.

The evidence discussed so far suggests that the method might be suitable for the measurement of ionized calcium and magnesium. When complexing agents were added to solutions calcium was excluded so enabling the investigation of magnesium complex formation in the

absence of competing ions. EHDP added to magnesium containing solutions had a very small but significant effect on eluted magnesium, in plasma it had no effect. Binding constants for EHDP-magnesium have not been determined; however in Chapter VI evidence will be presented that EHDP, added to plasma at lower concentrations than those used here, caused a significant fall in ultrafiltrable magnesium. It seems, therefore, that at the EHDP concentrations used in this study, large decreases in ionized magnesium went undetected by the method.

EDTA and citrate added to magnesium containing solutions also had no effect on measured ionized magnesium. In these cases binding constants are known, allowing calculation of expected ionized concentrations. The amount of magnesium bound by EDTA is approximately equal to the EDTA concentration. Citrate binds magnesium much less strongly. It is apparent from figure 3.4 that the method does not distinguish between magnesium ions and magnesium bound by EDTA or citrate. One must conclude that in the presence of EDTA, citrate or EHDP the method did not measure true ionized magnesium. Though the effect of complexing agents on measured ionized calcium was not studied, it seems likely that complexed calcium would also be adsorbed by ion exchange strips.

Frizel _et al_ (1967) also used a low capacity ion exchange strip to measure ionized calcium and magnesium, and investigated the effect of ligands on measured free ionic concentrations. When citrate was added to a standard, a significant reduction in adsorbed calcium and magnesium was observed. However free citrate calculated from their data was 400% greater than a value predicted from published binding constants. It appears, therefore, that their method does not accurately measure ionized magnesium either. It

may be that the problem encountered with Heaton's (1967) method may be of more general application to methods involving low capacity ion exchange strips.

In addition to the adsorption of cations by the dye, relatively large amounts of the solution under investigation may be trapped within the pores of the visking tubing. Typically, wet visking tubing contains 50% by weight of trapped solution. This might explain why the measured "ionized" magnesium and calcium in plasma is invariably less than the total, since protein would not penetrate the visking tubing pores and so protein bound cations would remain free in the sample. This mechanism would not explain the interference effects seen which are probably due, as expected, to cationic binding sites.

In conclusion, it is likely that measurement of ionized magnesium as a routine experimental procedure will have to await the production of a suitable ion specific electrode or the production of more suitable ion exchange materials.

Evaluation of the Nova-2 analyser for measuring calcium ions

1. Variability of the method

Within-run variability was assessed by collecting heparinised plasma and analysing it repeatedly. The coefficient of variation was 0.9% (n=11).

Day to day variability was assessed by analysing the 1.5 mM calcium standard (American Hospital Supply UK Ltd) over a period of one month, at no less than daily intervals. The coefficient of variation was 1.8% (n=23).

2. Linearity of the method

Standards containing 0.5-2.5 mM calcium were analysed and the electrode emf recorded. 0.5, 1.5 and 2.5 mM were American Hospital Supply external standards; 1.0 and 2.0 mM were American Hospital Supply internal standards; 0.75 mM calcium was obtained by mixing 0.5 and 1.0 mM standards.

Figure 3.5 shows that electrode emf was linearly related to log calcium concentration over the studied range.

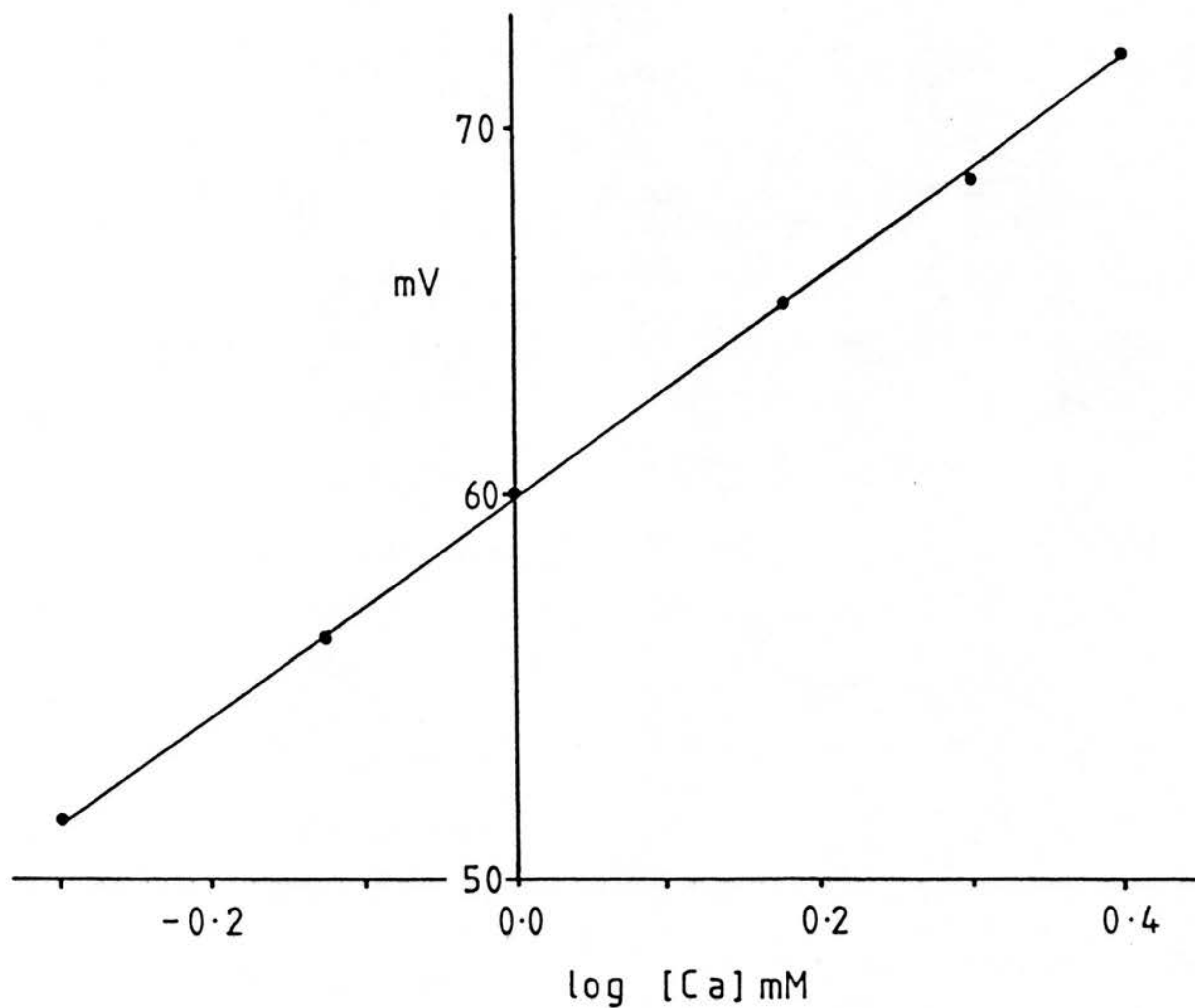


Figure 3.5 The electrode emf produced by the Nova-2 calcium ion analyser in response to changes in log [calcium ion] of aqueous solutions.

3. Recovery of added calcium chloride

Aliquots (0.05 ml) of calcium chloride solutions (0.18, 0.36 or 0.54 M) were placed in 5 ml syringes which were then filled with 5 ml of heparinised blood. Ionized calcium was measured, and plasma separated for calcium assay by AAS.

Addition of calcium chloride raised total plasma calcium concentration by 2.95, 6.01 or 9.03 mM. The increments in ionized calcium, expressed as a % of the increase in total calcium, were, respectively: 51.5 $\pm$ 3.93%, 48.8 $\pm$ 3.01% and 49.9 $\pm$ 3.65% (n = 4).

4. Removal of calcium from plasma with EDTA

In an experiment to be described on page 144, EDTA was added to heparinised plasma to give concentrations of 0.3-3.0 mM EDTA.

Ionized calcium in untreated plasma was 1.21 mM. Ionized calcium was reduced after addition of EDTA. On a molar basis the reduction in ionized calcium was approximately half the EDTA concentration (table 3.3).

Table 3.3

The decrease on ionized calcium, as measured by the Nova-2 calcium ion analyser, following addition of EDTA to sheep plasma

Plasma [EDTA] mM	mM change in ionized calcium
0.3	0.18 ± 0.03
0.6	0.31 ± 0.01
0.9	0.45 ± 0.02
1.2	0.59 ± 0.03
1.8	0.85 ± 0.03
2.4	1.09 ± 0.04
3.0	1.18 ± 0.04

Values are means ± standard errors for 5 observations. Control blood contained 1.21 mM ionized calcium.

5. The effect of blood heparin concentration on measurements of ionized calcium

Aliquots (0.049 ml) of lithium heparin solutions containing 625, 1250, 2500 or 5000 i.u. ml^{-1} added to 2 ml blood samples gave final heparin concentrations of 15-123 i.u. ml^{-1}. Ionized calcium was measured in serum and in the heparinised blood. The small dilution error was accounted for by multiplying the value for serum calcium by 0.975.

Heparin reduced ionized calcium (figure 3.6). Up to 30 i.u. ml^{-1} heparin, the rate of fall of ionized calcium was approximately linear. Calculation of the gradient of this portion of the line reveals that ionized calcium decreased 1% per 7 i.u. ml^{-1} increase in heparin concentration.

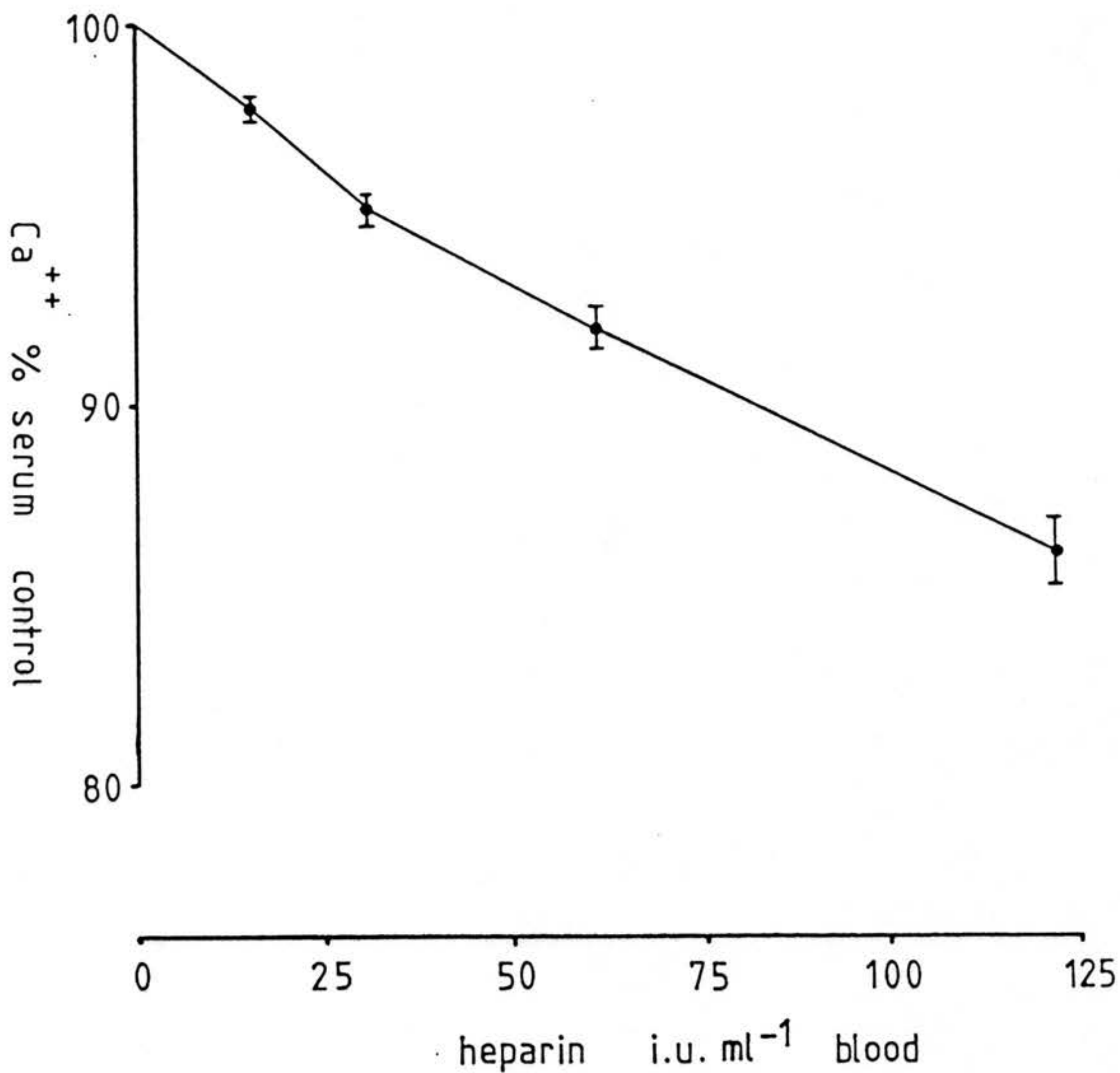


Figure 3.6 The effect of blood heparin concentration on ionized calcium as a % of serum controls. Each point is a mean and standard error of four observations. Control serum contained 1.25 mM calcium.

Discussion

The variability of this method was excellent and unlikely to be a significant source of error. The day to day analysis of 1.5 mM calcium standards gave a mean value of 1.55 mM, not significantly greater than 1.5. However Larsson and Ohman (1980) noted that calcium ion concentration measured with the Nova-2 analyser was consistently higher than simultaneous analyses made with an Orion SS-20 calcium electrode system. This may result from the different type of electrodes used in the two systems. Which of the two systems provides the more accurate estimate of ionized calcium remains uncertain.

Electrode emf was linearly related to log calcium ion concentration, as would be expected on theoretical grounds. Only 50% of calcium chloride added to plasma was recovered as ionized calcium. This was probably because of uptake of calcium by endogenous ligand binding sites. Similarly, when EDTA was added to blood, only 50% of the removed calcium came from the ionized fraction. These findings are as would be expected since normally approximately 50% of plasma calcium is ionized, plasma proteins serving to buffer most of the remainder of the removed or added calcium.

Heparin complexes calcium and so interferes with estimations of ionized calcium (Moore, 1970; Ladensen and Bowers, 1973; Boyd and Williamson, 1980.) The use of serum would have avoided this problem, but was impracticable because of the low yield. Consequently, it was necessary to use heparinised blood so as to provide sufficient material for the number of analytical procedures required. It was therefore necessary to quantify the potential

error due to heparin.

For measurement of ionized calcium, a blood heparin concentration of 10 i.u. ml^{-1} was used. The gradient of the linear portion of figure 3.6 indicates that this would reduce ionized calcium by approximately 1.5%; less than the day to day variability of the method. However the error is large enough to be detected within a run of analyses, so heparin concentration should be closely controlled.

The only way this source of error could be eliminated is by using serum. However the delay incurred by the preparation of serum would be such that even greater errors would be likely to occur as a result of pH changes (Siggaard-Andersen, 1961; Pedersen, 1971a). Alternatively the blood heparin concentration could be further reduced. However the risk of blood clotting and so damaging the electrodes would be increased. In view of the ease with which the electrode seemed to block, this risk was unacceptable.

The Nova-2 calcium ion analyser seems to provide an acceptable method of measuring ionized calcium providing that the required care in handling blood samples is exercised.

III

Evaluation of the method of preparing ultrafiltrates

1. Variability of the method

Thirty seven samples of plasma were ultrafiltered in duplicate and calcium and magnesium determined by AAS in the ultrafiltrates. The average coefficient of variation between duplicates was 3.55% for calcium and 3.34% for magnesium.

2. Protein permeability of the visking tubing

The presence of protein in the ultrafiltrate was tested for by adding a few drops of trichloro-acetic acid (10% w/v) to the ultrafiltrate. The formation of a faint opalescence was taken as a positive result. This method was found to be extremely sensitive, slight wetting of the outside of the sac was sufficient to give a positive reaction.

Initially a large number of samples were shown to be contaminated with protein. Great care was required during filling of the visking tubing sacs, ensuring that the outside of the sac was not wetted. Filling too quickly could easily cause the sac to overflow, the plasma being drawn up between the closely apposed surfaces by capillarity. When the required care was appreciated, it was found that the problem was readily eliminated. Out of 146 samples of ultrafiltrate tested, only 3 were positive.

3. Changes in pH and pCO_2 following ultrafiltration

Blood was collected from sheep and pH and pCO_2 measured immediately. Plasma was then separated, some was ultrafiltered and some stored at 4°C. After ultrafiltration pH and pCO_2 were measured in retentate and in stored (control) plasma.

Ultrafiltration caused a rise in pH, and a fall in pCO_2 (table 3.4). Similar changes also occurred during the time taken to separate plasma from blood.

Table 3.4

The changes, induced by ultrafiltration, in pH and pCO_2 in sheep plasma

	pH	pCO_2 mmHg
Blood, immediately after collection		
	7.42	44.7
Plasma, before and after ultrafiltration		
Control value	7.47	40.3
Mean change following ultrafiltration	+0.076	-6.08
Standard error	0.0028	0.68
P (cf. 0.0)	<0.001	<0.001

Values are means ± standard errors for 5 observations.

4. <u>The effect of temperature on ultrafiltrable calcium and magnesium</u>

Plasma was ultrafiltered at 37°C or 20°C and calcium and magnesium determined in both ultrafiltrate and plasma. Ultrafiltrable calcium, as a % of total, was significantly higher at 20°C than at 37°C ($P<0.001$). Magnesium was similarly affected ($P<0.0001$; table 3.5).

5. <u>The Donnan equilibrium: Measurement of ultrafiltrate/plasma sodium, potassium and EDTA concentration ratios</u>

Plasma was ultrafiltered and then sodium and potassium concentration determined in both ultrafiltrate and plasma. EDTA partition was measured in plasma and ultrafiltrates collected during intravenous infusion of ^{14}C-EDTA (see Chapter IV for experimental details).

The ultrafiltrate/plasma ratios were: for sodium, 0.992 ± 0.005 (n = 5); for potassium, 1.004 ± 0.005 (n = 5); and for EDTA, 1.089 ± 0.025 (n = 16). The ratios differed significantly from 1.000 for both sodium ($P<0.05$) and EDTA ($P<0.01$).

Table 3.5

Ultrafiltrable calcium and magnesium, as % of total, measured after preparation of ultrafiltrates by centrifugation at two temperatures

Measured cation	37°C	20°C	
Calcium	50.1% ± 1.2 (11)	58.9% ± 2.0 (10)	$P<0.001$
Magnesium	72.3% ± 1.7 (11)	77.7% ± 0.7 (10)	$P<0.0001$

Values are expressed as % of the total calcium and magnesium concentrations in the samples, they are shown as means ± standard errors (number of observations).

Discussion

When the method was first used, the visking tubing was washed, then stored in a solution containing 150 mM NaCl, 4 mM KCl, 1.4 mM $CaCl_2$, and 0.6 mM $MgCl_2$, ultrafiltrates were then prepared using moist tubing. This appeared to work perfectly well when measuring "normal" values of ultrafiltrable calcium and magnesium. However the wet visking tubing held approximately its dry weight of solution. Since only 0.2-0.3 ml of ultrafiltrate was produced, and the visking tubing contained 0.2 ml of solution, it was probable that the ultrafiltrate was seriously contaminated.

This problem was overcome by adopting the procedure of Pedersen (1971b) for producing dry visking tubing. In addition, the use of dry tubing facilitated filling of the membrane sacs. Pinholing of the tubing was not found to be a problem as analysis of ultrafiltrates for protein showed. However very intensive washing was required, otherwise traces of magnesium and calcium were concentrated in the tubing during drying causing a significant rise in ultrafiltrate calcium and magnesium.

In addition to the investigations described, the effect of prolonged ultrafiltration was briefly examined. Over several hours of centrifugation time did not significantly change ultrafiltrable calcium and magnesium.

The pH change which occurred during ultrafiltration is likely to have some effect on measured ultrafiltrable calcium and magnesium. Pedersen (1971a) found that serum pH decreased by 0.06-0.08 pH units during ultrafiltration. Detailed study of the effects of bicarbonate, pCO_2 and pH on serum calcium fractions showed that increasing pH from 7.0 to 8.0 decreased the ultrafiltrable calcium

by 40% (Pedersen, 1971a). Over this range the increase in protein binding was linearly related to pH. In the present investigation the increase in pH from the time of collection of blood to completion of ultrafiltration was 0.12. The expected decrease in ultrafiltrable calcium was therefore 5%. On the assumption of linearity (Pedersen, 1971a), the data of Robertson and Peacock (1968) would suggest an effect of similar magnitude.

Siggaard-Andersen (1961) showed that at 38°C, under anaerobic conditions, blood pH fell linearly by 0.066 pH units per hour for four hours after collection. This effect was a function of time and could only be substantially reduced by storing at 4°C. The pH change shown in table 3.4 was in the opposite direction and was probably the result of CO_2 loss, but since approximately 3 hours elapses between blood collection and completion of ultrafiltration, the observed change was apparently smaller than that described by Siggaard-Andersen (1961). It therefore seems unlikely that the pH change in the present method could be substantially reduced.

Pedersen (1971a) found that cooling serum from 37°C, to 22°C caused pH to increase from 7.40 to 7.57. From his data he calculated that protein binding of calcium should decrease by 7%. In this study the effect of temperature was very similar (8.8%). The data of Robertson et al (1968) are also in good agreement.

The Donnan ratio is approximately 0.90 for divalent cations in plasma. Thus ultrafiltrable calcium and magnesium might be expected to be only 90% of their total plasma levels even in the absence of protein binding. In addition, ultrafiltrate/plasma ratios are also influenced by the plasma water factor. Plasma proteins effectively dilute the plasma water and so reduce the concentration of substances in plasma compared to ultrafiltrates. Although the

Donnan equilibrium may influence the formation of ultrafiltrate, the extent to which it does so is not clear. Walser (1969) did not consider that equilibrium conditions would be reached. Pedersen (1972b) could not detect such shifts in his method, contrary to the results presented here. However it seems unlikely that the electrical and concentration gradients across the membrane have absolutely no influence on the composition of ultrafiltrates.

In the present investigation, ultrafiltrate/plasma ratios for sodium and potassium were very close to 1.00. This suggested that for monovalent cations Donnan and plasma water factors were acting equally but in opposite directions. In the case of anions the Donnan effect and the plasma water factor will be additive since they both act to increase the measured concentration in the ultrafiltrate. In blood, EDTA is largely in the form of the calcium chelate which exists as a divalent anion (Schwartzenbach, 1957). The Donnan ratio for a divalent anion is 1.10, the plasma water factor is approximately 5%. The concentration of divalent anions would thus be expected to be 15% higher in the ultrafiltrate than in plasma; greater than the observed gradient. Thus it seems that although the plasma water factor and the Donnan effect appreciably altered the partition of anions across the semipermeable membrane, equilibrium was not reached. Similarly, it seems likely that divalent cation concentrations will be appreciably lower in ultrafiltrates than in plasma even in the absence of protein binding, although probably somewhat less than the 5% expected at equilibrium.

In studies of the chemistry of calcium and magnesium binding it would be necessary to take account of Donnan shifts, but since they are equally likely to occur *in vivo* as *in vitro,* the presence of

such shifts does not necessarily constitute a disadvantage when attempting to determine glomerular filtrable calcium and magnesium. Since this was the primary area of interest in the studies described in this thesis, no correction factors were necessary.

CHAPTER IV

THE EFFECT OF ETHYLENE DIAMINE TETRAACETIC ACID (EDTA) INFUSIONS ON THE PLASMA FRACTIONS AND URINARY EXCRETION OF CALCIUM AND MAGNESIUM IN SHEEP

Summary

1. EDTA containing tracer ^{14}C-EDTA was infused intravenously into sheep. Blood was collected every half hour and urine every hour, before, during and after the infusion. Total, ultrafiltrable and ionized calcium concentrations, and total and ultrafiltrable magnesium were determined in plasma. Plasma EDTA concentration, blood pH, endogenous creatinine clearance and the urinary excretion of calcium, magnesium and EDTA were also measured.

2. Following EDTA administration, ultrafiltrable plasma calcium concentration increased and ionized calcium decreased whilst the total was unchanged. The net increase in plasma complexed calcium was approximately equimolar with the EDTA concentration.

3. Calcium excretion also increased despite unchanged creatinine clearance. The increments in urinary EDTA and calcium were approximately equimolar.

4. The effect of EDTA on the measured plasma fractions of magnesium and calcium were similar; ultrafiltrable plasma magnesium increased, whilst the total remained unchanged. Unlike calcium, urinary magnesium excretion did not increase significantly.

5. The renal clearance of EDTA and endogenous creatinine were similar.

6. The effects of EDTA on plasma and urinary calcium are consistent with the formation in plasma of Ca-EDTA complexes which are then eliminated by glomerular filtration without tubular secretion or reabsorption. These findings are largely in agreement with previous studies, however the stoichiometric relationship between urinary calcium and EDTA has not been seen previously. The high urinary pH of herbivores probably accounts for this difference, since it results in enhancement of EDTA-calcium binding.

Introduction

The metabolism of EDTA has been studied in a number of species including human, dog and rat, but has not, so far, been studied in sheep. Soffer et al (1961) infused EDTA into human subjects and measured the concentrations of plasma calcium fractions. Total plasma calcium did not change but complexed calcium increased considerably. This effect was attributed to the presence of EDTA chelated calcium in plasma. Parfitt (1969) also obtained similar results in man.

EDTA is excreted by glomerular filtration in the dog (Forland et al, 1966). In the rat 95-98% of an administered dose of EDTA is eliminated within six hours (Foreman et al, 1953). Studies have demonstrated that EDTA infusion induces hypercalciuria in man (Spencer et al, 1952; Holland et al, 1953; Levitt et al, 1958), dog (Chen et al, 1955a) and rat (Rubin et al, 1956-7). However the increment in urinary calcium excretion seems to be considerably less than that predicted on the basis of stoichiometric binding (Spencer et al, 1952; Rubin et al, 1956-7; Parfitt, 1969). Stop-flow studies in dogs have demonstrated that the calcium concentration in samples corresponding to the distal tubule is greatly increased by EDTA (Grollman et al, 1963) indicating depression of calcium reabsorption in the distal tubule.

Methods

The pH of Na_2EDTA solution was adjusted to 7.40 with sodium hydroxide and a 3.76% solution, approximately isotonic with plasma, was made. Sheep were equipped with Foley urinary and PVC jugular catheters. They were then infused with a priming dose of 2.5 micro-mol $min^{-1}kg^{-1}$ EDTA for half an hour followed by a maintenance dose of 0.7 micro-mol $min^{-1}kg^{-1}$ for two hours. The Harvard infusion pump was set to infuse at 0.6 ml min^{-1}. EDTA solution was diluted with saline to provide the appropriate dose. Two preliminary infusions of unlabelled EDTA were made using this dosage regime; having established that a measurable effect occurred four further infusions of EDTA, containing 8 micro-Ci of ^{14}C-EDTA (108 Ci mol^{-1}; Radiochemical Centre, Amersham) were made. Blood samples were collected every 30 minutes and urine for periods of one hour except for the half hour period during administration of the priming dose.

In these experiments it was attempted to induce a mild diuresis by the administration of four litres of water by naso-pharyngeal tube 90 minutes before the start of urine collection. EDTA was administered after a two hour control period of urine and blood collection. When the infusion was complete, blood and urine collection continued for a further four hours. Plasma and blood fractions of calcium and magnesium were measured. Creatinine and EDTA were also determined.

Results

Only the data from the four sheep infused with ^{14}C-EDTA are reported. The other two animals showed similar responses, but were excluded to allow comparisons between EDTA and calcium concentrations in blood and urine.

During EDTA infusion, plasma ionized calcium concentration fell ($P<0.001$) whilst the ultrafiltrable calcium fraction increased ($P<0.01$) and the total did not change significantly (figure 4.1). The increase in complexed calcium during the period of EDTA infusion was 0.49 ± 0.06 mM ($n = 8$) and was not significantly different from the EDTA concentration observed in the same samples (0.47 ± 0.04, $n = 8$).

The urinary excretion of calcium increased 100 fold ($P<0.001$; figure 4.2). The increment in the urinary excretion of calcium was very similar to that of EDTA and the correlation highly significant ($r = 0.94$, $P<0.001$).

The ultrafiltrable fraction of plasma magnesium also increased ($P<0.05$; figure 4.3) but total plasma magnesium did not change. Total plasma calcium and magnesium were correlated during the control period ($r = 0.59$, $P<0.01$) and during the infusion period ($r = 0.86$, $P<0.001$).

The urinary excretion of magnesium did not change significantly (table 4.1). No significant changes in blood pH (figure 4.4) or H^+ excretion (table 4.1) were seen. Endogenous creatinine clearance did not change significantly (table 4.2). It was similar to the simultaneous EDTA clearance but the SEM of EDTA clearance during each collection period was large.

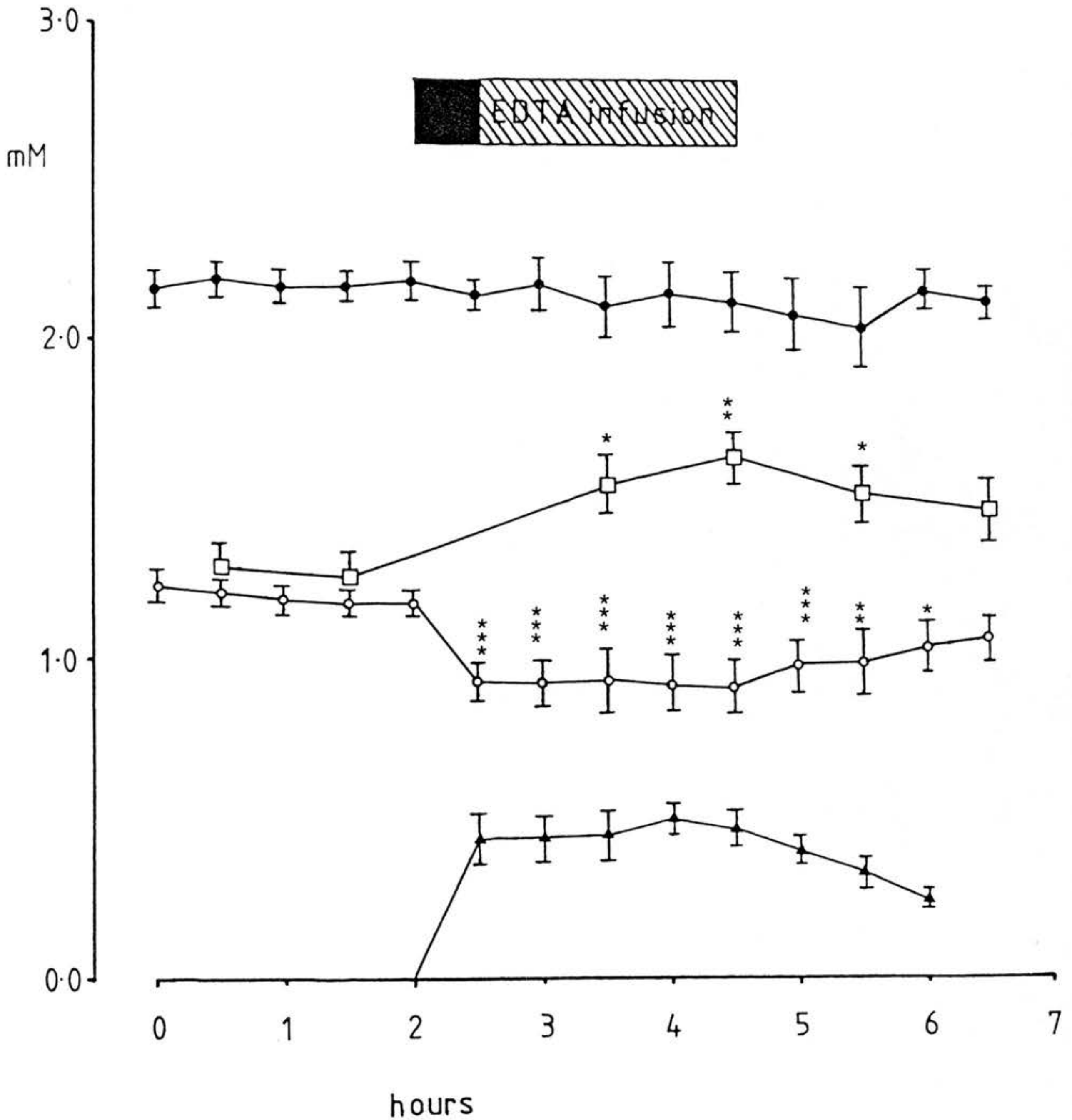


Figure 4.1 The effect of EDTA infusion in four sheep on the concentration of total (closed circles), ultrafiltrable (squares) and ionized (open circles) plasma calcium, and plasma EDTA concentration (triangles). EDTA was infused at 7 micro-mol $min^{-1}kg^{-1}$ for 120 minutes (shown hatched), following a 30 minute priming dose at 25 micro-mol $min^{-1}kg^{-1}$. Values of calcium concentrations were compared with their pre-infusion controls: ***, P<0.001; **, P<0.01; *, P<0.02.

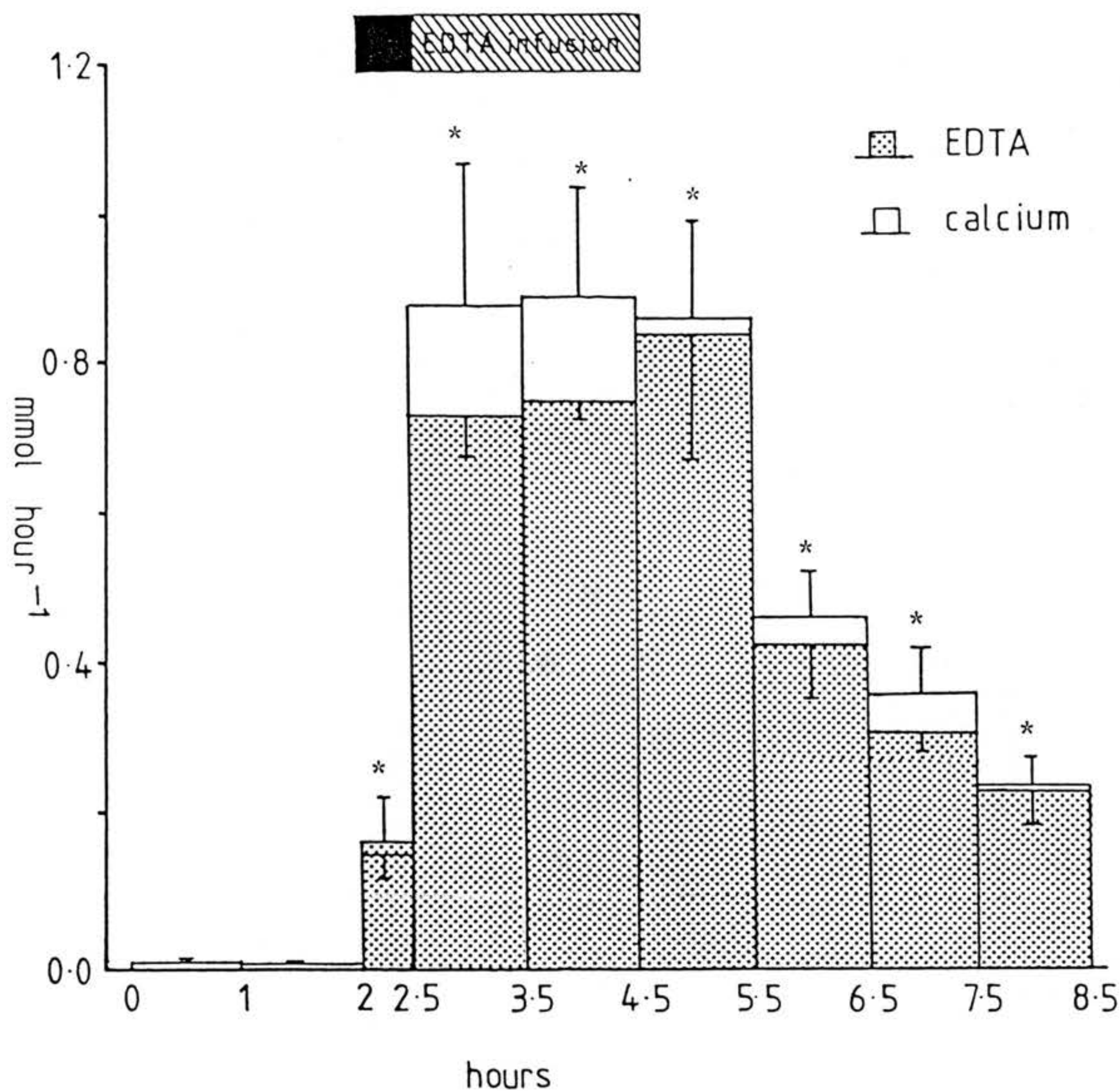


Figure 4.2 The effect of EDTA infusion in four sheep on the urinary excretion of EDTA and calcium. EDTA was infused at 7 micro-mol $min^{-1}kg^{-1}$ for 120 minutes (shown hatched), following a priming dose of 25 micro-mol $min^{-1}kg^{-1}$ for 30 minutes. Values of calcium concentrations were compared with their pre-infusion controls: *, $P<0.001$.

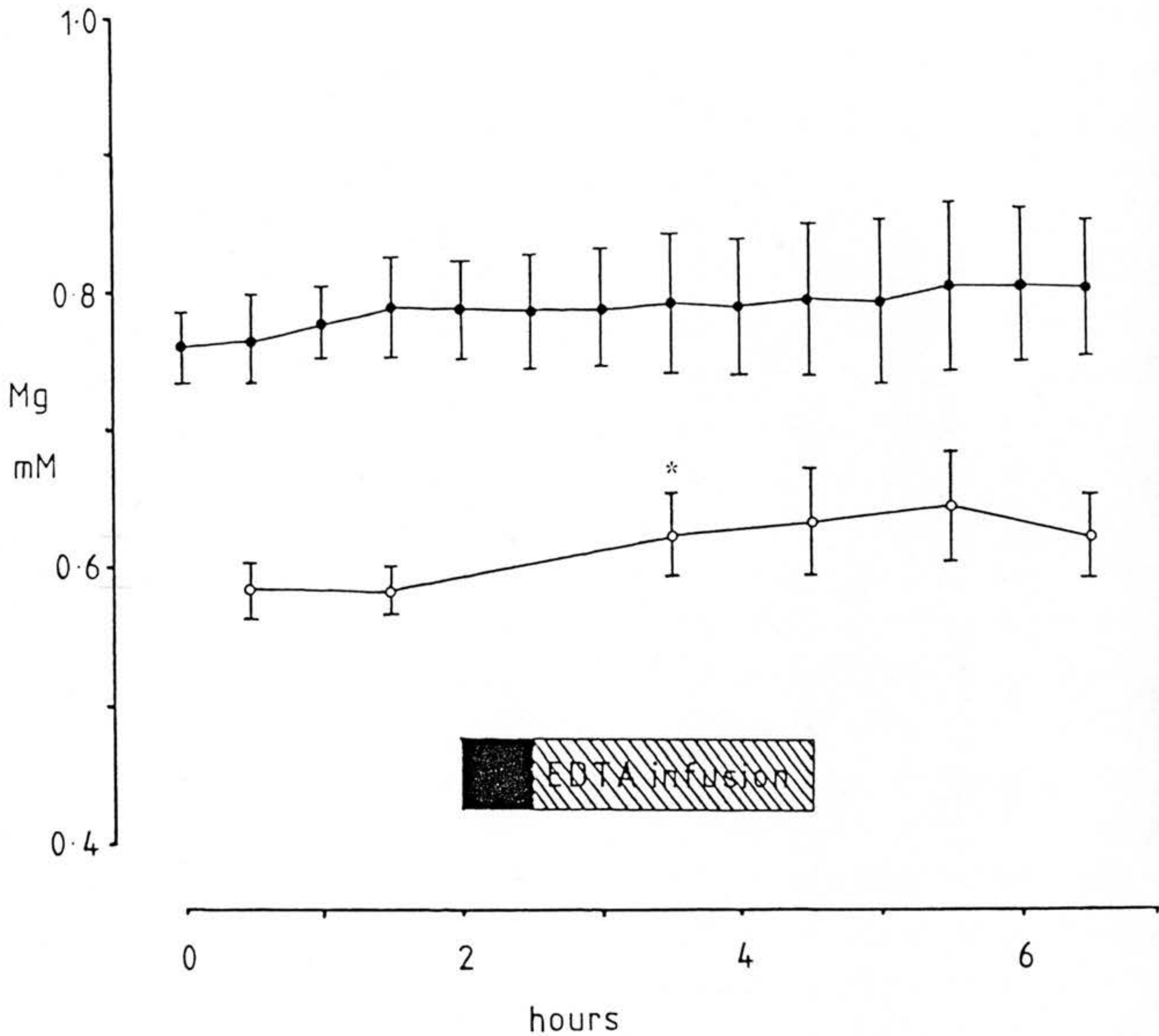


Figure 4.3 The effect of EDTA infusion in four sheep on the concentration of total (closed circles) and ultrafiltrable (open circles) plasma magnesium. EDTA was infused at 7 micro-mol $min^{-1}kg^{-1}$ for 120 minutes (shown hatched), following a 30 minute priming dose at 25 micro-mol $min^{-1}kg^{-1}$. Values were compared with their pre-infusion controls: *, $P<0.05$.

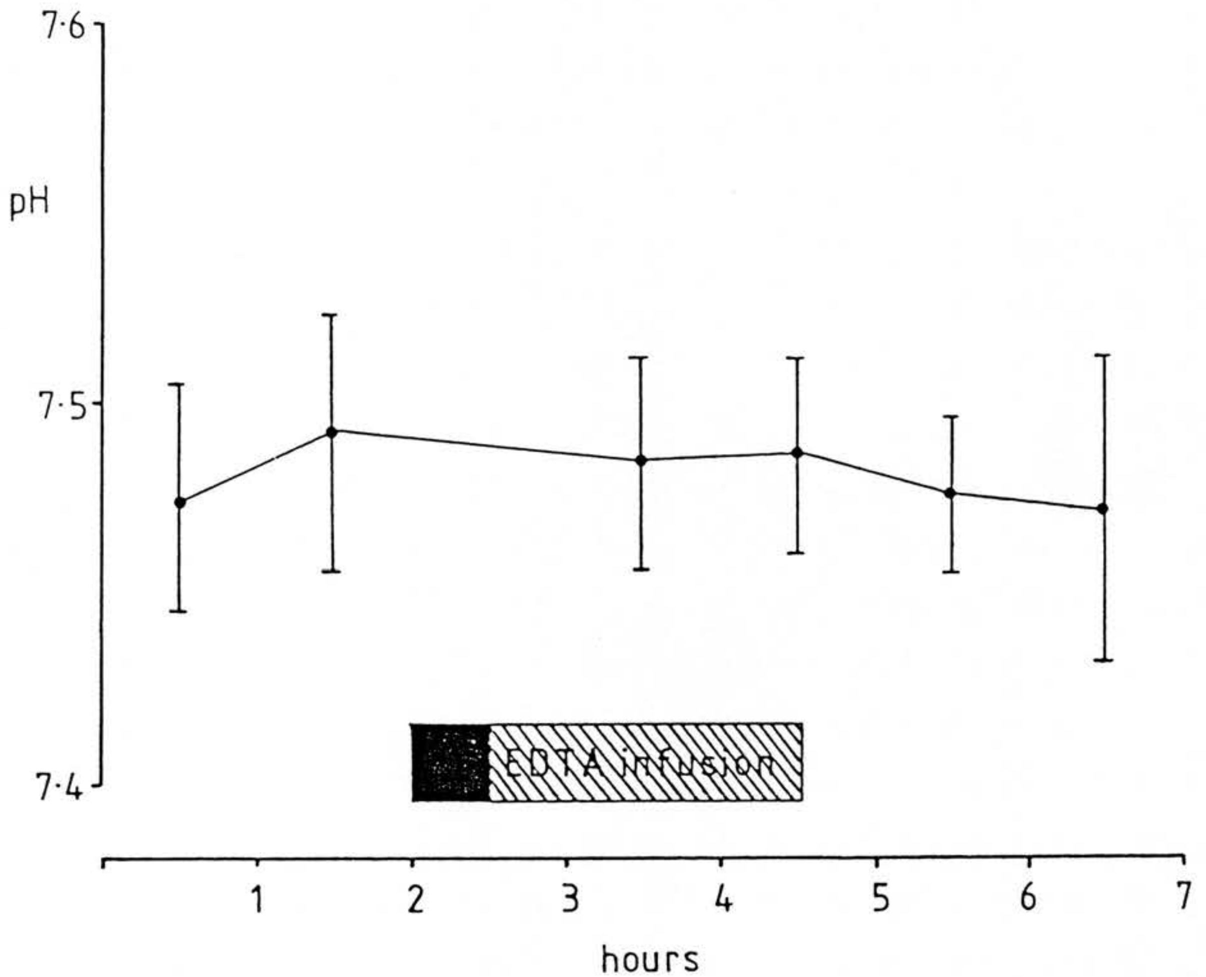


Figure 4.4 The effect of EDTA infusion in four sheep on blood pH. EDTA was infused at 7 micro-mol $min^{-1}Kg^{-1}$ for 120 minutes (shown hatched), following a 30 minute priming dose at 25 micro-mol $min^{-1}Kg^{-1}$. Values were compared with their pre-infusion controls.

Table 4.1

The effect of EDTA infusion on the excretion of H^+ and magnesium in the sheep

Collection period hours	Magnesium mmol $hour^{-1}$	H^+ nmol $hour^{-1}$
	Control period	
0-1	0.13 ± 0.08	13.0 ± 10.5
1-2	0.13 ± 0.10	9.8 ± 7.1
EDTA infusion rate: 25 micro-mol $kg^{-1}min^{-1}$		
2-2.5	0.17 ± 0.12	12.9 ± 10.2
EDTA infusion rate: 7 micro-mol $kg^{-1}min^{-1}$		
2.5-3.5	0.17 ± 0.06	11.7 ± 10.4
3.5-4.5	0.11 ± 0.05	11.6 ± 10.4
	Post-infusion period	
4.5-5.5	0.11 ± 0.04	10.7 ± 8.9
5.5-6.5	0.18 ± 0.10	17.1 ± 10.8

Each value is a mean ± standard error of four observations. Magnesium and H^+ excretion did not differ significantly in the control, infusion, or post-infusion periods.

Table 4.2

A comparison of the renal clearances of endogenous creatinine and ^{14}C-EDTA in the sheep

Collection period	^{14}C-EDTA clearance	Endogenous creatinine clearance
hours	ml min^{-1}	ml min^{-1}
	Control period	
0-1		41.8 ± 8.1
1-2		42.5 ± 7.0
EDTA infusion rate: 25 micro-mol $kg^{-1}min^{-1}$		
2-2.5		46.6 ± 11.1
EDTA infusion rate: 7 micro-mol $kg^{-1}min^{-1}$		
2.5-3.5	29.6 ± 4.6	38.6 ± 7.0
3.5-4.5	27.3 ± 4.3	34.8 ± 6.5
	Post-infusion period	
4.5-5.5	40.9 ± 16.0	38.5 ± 6.2
5.5-6.5	32.4 ± 11.7	29.8 ± 3.5
7.5-8.5	44.6 ± 17.8	43.1 ± 8.3
8.5-9.5	40.7 ± 12.1	40.3 ± 6.9

Each value is a mean ± standard error of four observations. The clearances of EDTA and endogenous creatinine were not significantly different during each period. Endogenous creatinine clearance did not differ significantly in the control, infusion, or post-infusion periods.

Discussion

The effects of EDTA on plasma and urinary calcium were as expected. The complexed calcium fraction in blood increased, the increase was equimolar with the EDTA concentration in the same samples. Similarly, the excretion of EDTA was accompanied by a large increment in urinary calcium. In the first two experiments urinary ultrafiltrable calcium was determined. This confirmed that the increment in urinary calcium was indeed ultrafiltrable. It seemed that the increase in complexed calcium in blood was due to the formation of EDTA-calcium complexes which were then eliminated by the kidney. The effect on complexed calcium seemed to involve an increase in ultrafiltrable plasma calcium and a decrease in the ionized fraction.

These changes are as expected on the basis of previous studies (Spencer *et al*, 1952; Soffer *et al*, 1961). The effect of EDTA on the excretion and plasma fractions of calcium was due to the formation in blood of EDTA-calcium complexes which were then eliminated by glomerular filtration without further reabsorption or secretion. The increase in calcium excretion was primarily due to the non-availability of complexed calcium for tubular reabsorption. However, in addition, since GFR was unchanged and ultrafiltrable calcium increased, the filtered load of calcium entering the nephron also increased. This would also exacerbate renal calcium loss.

Unlike previous studies in which the increment in urinary calcium was considerably less than the increment in EDTA, this study obtained similar increments in both substances. This may have resulted from the relatively alkaline urine produced by ruminants

which would enhance calcium binding.

The clearance of endogenous creatinine and EDTA were similar. This suggests that in the sheep EDTA was primarily excreted by glomerular filtration, as it is in the dog (Forland *et al*, 1966). However, since the clearance of EDTA was very variable, the evidence is not completely conclusive.

The increase in ultrafiltrable magnesium (approximately 0.05 mM) during EDTA infusion was unexpected. It is unlikely to have been due to a reduction in plasma magnesium protein binding since blood pH, the main determinant of protein binding, did not change. It may be that the increase in plasma ultrafiltrable magnesium was a result of the formation of EDTA-magnesium complexes. From published EDTA binding constants, the maximum concentration of magnesium likely to be bound is 0.005 mM, only 10% of the observed effect. However the value of such constants is uncertain since it has been found that under physiological conditions the EDTA-calcium constant is an order of magnitude less than published values (Kim and Padilla, 1978); it would not be surprising if EDTA-magnesium constants err similarly. Clearly, corroborative evidence is required to justify the conclusion that EDTA complexes magnesium *in vivo*.

Since calcium and magnesium compete for renal tubular reabsorption, by complexing calcium EDTA might be expected to enhance magnesium reabsorption as a result of reduced ionized calcium in the renal tubule. However, since EDTA infusion did not decrease magnesium excretion, the experiments do not indicate that competition occurred. Previous studies have demonstrated competition during periods of raised plasma calcium and magnesium (Samiy *et al*, 1960; Massry *et al*, 1969). It may be that in normal

circumstances competition between calcium and magnesium for tubular reabsorption is not of physiological importance in the homeostasis of these ions. Such conjectures are clearly not justified if in fact EDTA complexes significant amounts of magnesium, since this, in itself, would cause hypermagnesuria. The use of a ligand such as EGTA, which shows considerably greater selectivity for calcium than does EDTA might clarify this point.

Despite the hypercalciuria observed during EDTA infusion, in this and other studies total plasma calcium often remained constant (Spencer et al, 1952; Soffer et al, 1961). It seems, therefore, that the plasma calcium pool is being replenished from some external store. The most likely source is bone. Ramberg, Mayer, Kronfeld, Aurbach, Sherwood and Potts (1967) showed that parathyroid hormone (PTH) secretion was active within 15 minutes of commencing EDTA infusions in cattle. However it is unlikely that PTH is involved in the maintenance of plasma calcium during the preliminary stages of EDTA infusion since PTH mediated bone resorption is not active until 70-80 minutes following administration (Parfitt, 1969). In addition PTH may cause a transient hypocalcaemia during the first 20 minutes post-administration (Parsons and Robinson, 1971), this was certainly not observed during EDTA infusion.

Rodan, Lieberman, Paran and Anbar, (1967) found that when EDTA was administered to the dog hind limb in which the blood supply was isolated from the rest of the animal, plasma calcium concentration in the isolated limb rose. They calculated that the available calcium was approximately 1/1000 of total bone calcium. This mechanism may be responsible for the maintenance of plasma calcium during the early stages of EDTA infusion.

In conclusion, in the sheep EDTA acts as a classical chelating agent, as previously described in other species. The effect of EDTA on plasma calcium fractions resulted in enhanced renal excretion of calcium. The failure significantly to alter total plasma calcium suggests that bone has the ability rapidly to release calcium into the extracellular fluid.

CHAPTER V

THE FORMATION OF CALCIUM COMPLEXES BY BOROGLUCONATE IN VITRO AND DURING CALCIUM BOROGLUCONATE INFUSION IN SHEEP

Summary

1. The calcium binding ability of calcium borogluconate, a drug widely used for the treatment of hypocalcaemia, does not seem to have been investigated. The effect of sodium and calcium borogluconate on ionized calcium was studied *in vitro*. Calcium borogluconate was then infused into sheep. Blood was collected every fifteen minutes and urine for periods of one hour. Total, ultrafiltrable and ionized calcium, and total and ultrafiltrable magnesium concentrations were determined in plasma. Plasma pH and pCO_2, endogenous creatinine clearance and the urinary excretion of calcium, magnesium and hydrogen ion were determined.
Finally, the effect of subcutaneous administration of calcium and sodium borogluconates on total plasma calcium and magnesium concentrations was examined in sheep.

2. When equimolar amounts of calcium as the borogluconate or chloride were added to blood *in vitro*, the chloride salt caused the greater increase in ionized calcium.

3. Addition to blood, *in vitro*, of either sodium borate (5 mM) or sodium gluconate (5 mM), caused small decreases (0.08 mM) in plasma ionized calcium. The two substances together had a synergistic action, causing a large decrease in ionized calcium (0.53 mM).

4. The effects of intravenous infusion of calcium borogluconate were:

(i) a large increase in both total and ultrafiltrable plasma calcium concentration;

(ii) a relatively small increase in plasma ionized calcium;

(iii) an increase in calcium excretion, accompanied by a fall in endogenous creatinine clearance post-infusion;

(iv) respiratory acidosis, developing during the infusion, and a fall in urinary pH post-infusion;

(v) a slight fall in total plasma magnesium concentration which may have resulted from competition between calcium and magnesium for renal tubular reabsorption (although magnesium excretion did not increase significantly).

5. Following subcutaneous administration of sodium borogluconate to sheep, total plasma calcium concentration decreased.

6. It is suggested that the effect of calcium borogluconate on urinary and plasma calcium fractions was due to the formation of calcium-borogluconate complexes. The hypocalcaemic effect of sodium borogluconate was probably due to decreased renal tubular reabsorption of complexed calcium. The *in vitro* studies showed that the calcium complexes formed involve both borate and gluconate anions.

Introduction

Crystallised calcium borogluconate solid appears to exist as a distinct chemical substance (Dryerre and Greig, 1935; Macpherson and Stewart, 1938). However the physico-chemical nature of calcium in an aqueous solution of the salt does not seem to have been investigated. Its beneficial attributes, as compared with the gluconate and chloride salts are its stability and non-irritant nature (Dryerre and Greig, 1935).

Gluconate causes hypercalciuria by depressing the renal tubular reabsorption of calcium (Howard et al, 1959; Bernstein et al, 1962; Dibona, 1971). Additionally, calcium gluconate is less effective than calcium chloride in raising plasma ionized calcium concentration (White et al, 1976). These effects are probably the result of formation of calcium complexes with reduced bio-availability.

In view of the widespread use of calcium borogluconate in veterinary practice and its chemical relationship to gluconate, which is believed to complex calcium, it was proposed to investigate the possible formation of calcium complexes in vitro. These experiments were followed by studies in vivo.

Methods and Results

1. The in vitro ionization of calcium borogluconate and calcium chloride in blood

Methods

Solutions of the calcium salts, borogluconate (Astra Chemicals Ltd) and chloride (BDH Analar Reagent) were prepared containing slightly in excess of 90 mM calcium. These two solutions were then analysed by AAS and slight volume adjustments made to provide two solutions containing 90 mM calcium.

Portions (0.1, 0.2 or 0.3 ml) of the calcium solutions were placed in 5 ml syringes which were then filled with 5 ml of heparinised sheep blood and thoroughly mixed. Ionized calcium was measured in these samples and in control blood containing no additional calcium. The procedure was carried out with blood from four animals. The pH of control blood and blood containing the largest amount of the two calcium solutions was also measured.

Results

Table 5.1 compares ionized calcium in blood after the addition of three concentrations of calcium as the borogluconate or as the chloride. At every concentration of calcium, ionized calcium was considerably lower in the presence of calcium borogluconate than it was in the presence of the chloride salt ($P<0.02$).

The pH of blood containing calcium chloride (7.33 ± 0.04) or calcium borogluconate (7.22 ± 0.03) was less ($P<0.002$) than the pH of control blood (7.40 ± 0.04). This is to be expected since calcium chloride is an acidic salt and calcium borogluconate

contains boric acid. The acidifying effect of calcium borogluconate was greater than that of calcium chloride (P<0.007).

Table 5.1

A comparison of ionized calcium concentrations after the addition of equimolar amounts of calcium borogluconate or calcium chloride to blood

Volume (ml) 90 mM calcium added to 5 ml blood	Ionized calcium (mM), with chloride	Ionized calcium (mM), with borogluconate	P, comparison of salts
0.0	1.26 ± 0.06	1.26 ± 0.06	
0.1	2.78 ± 0.18	2.32 ± 0.06	P<0.02
0.2	4.19 ± 0.14	2.76 ± 0.06	P<0.0001
0.3	5.77 ± 0.25	2.95 ± 0.06	P<0.0001

Values are means ± standard error of four observations. The ionized calcium in untreated blood was 1.26 ± 0.06 mM.

2. Ionized calcium in vitro after addition of sodium gluconate and sodium borate to blood

Methods

The following solutions were prepared: (1) 0.2 M sodium chloride (control); (2) 0.2 M sodium gluconate (Sigma); (3) 0.2 M boric acid; (4) 0.2 M sodium gluconate and 0.2 M boric acid. The solutions were adjusted to pH 7.40 with sodium hydroxide. Aliquots (0.05 ml) of each solution were placed in 2 ml syringes which were then filled to the 2 ml mark with heparinised sheep blood. Ionized calcium was then measured. The procedure was carried out five times with blood from different animals.

Results

Table 5.2 shows that sodium gluconate caused a 0.08 mM decrease in ionized calcium compared with saline controls ($P<0.02$), the effect of boric acid was similar although less significant ($P<0.06$). Together, sodium gluconate and boric acid had a large synergistic effect on ionized calcium, which fell by 0.53 mM ($P<0.0005$).

Table 5.2

The effect on ionized calcium concentration of the addition to blood of sodium gluconate, sodium borate, or both substances

Solution added to blood	Blood concentration	Measured ionized calcium	P, comparison of treated blood & saline
saline	5 mM	1.27 $\pm$ 0.03	
Sodium gluconate	5 mM	1.19 $\pm$ 0.03	$P<0.02$
Sodium borate	5 mM	1.19 $\pm$ 0.05	$P<0.06$
Sodium gluconate and sodium borate	5 mM 5 mM	0.74 $\pm$ 0.04	$P<0.0005$

Values are means $\pm$ standard errors of five observations.

3. The effect of calcium borogluconate infusion on the plasma fractions and urinary excretion of calcium and magnesium in sheep

Methods

Calcium borogluconate (60 ml, 40% w/v, Astra Chemicals Ltd) was diluted with 90 ml of saline (0.9% w/v sodium chloride). This prevented precipitation during the subsequent adjustment of pH to 7.40 with sodium hydroxide. Finally the solution was diluted to 170 ml with saline. Sheep were catheterised for infusion purposes and for urine and blood sampling as described in Chapter II. Blood was collected every 15 minutes, and urine for periods of one hour. The animals were infused intravenously at 2.75 ml min^{-1} with the calcium borogluconate solution. This provided a dose of 0.53 mmol min^{-1} calcium and thus a total dose of 31.7 mmol calcium; approximately a normal clinical dose for a sheep. Saline was infused at the same rate before and after the period of calcium administration.

Results

Figure 5.1 shows that during and after the infusion of calcium borogluconate large increases in both total and ultrafiltrable calcium occurred accompanied by a smaller increase in ionized calcium.

When total plasma calcium concentration was plotted against ultrafiltrable and ionized calcium, as in figure 5.2, it became much more apparent that the increment in total calcium was largely due to an increase in the non-ionized, ultrafiltrable component of plasma calcium. The gradient of the calculated regression of

ionized on total plasma calcium was less than the gradient of the regression of ultrafiltrable on total calcium ($P<0.01$).

Total plasma magnesium fell gradually almost from the start of the calcium infusion (figure 5.3) but was not significantly lower than the control value until 45 minutes after the calcium infusion had ceased ($P<0.02$). The gradient of the regression of plasma magnesium on time, calculated from the time the infusion started, was significantly different from zero (t-test, $P<0.001$). Ultrafiltrable plasma magnesium, as a total or as a percentage of total, was not significantly altered by calcium infusion.

Infusion of calcium borogluconate (pH 7.40) was associated with respiratory acidosis (figure 5.4); blood pH decreased and pCO_2 increased. This resulted in an increase in bicarbonate concentration. Urinary pH was not significantly different during the period of infusion (7.10 ± 0.51) than during the control period (8.13 ± 0.17), however by the post infusion period it was significantly lower ($P<0.02$, 6.18 ± 0.56) than control. When calculated in terms of H^+ excretion the change, although large, was not significant (figure 5.5). Calcium borogluconate infusion was followed by a reduction in GFR, as measured by endogenous creatinine clearance (figure 5.5). The urinary excretion of calcium increased during the post infusion period but no significant change in the urinary excretion of magnesium occurred.

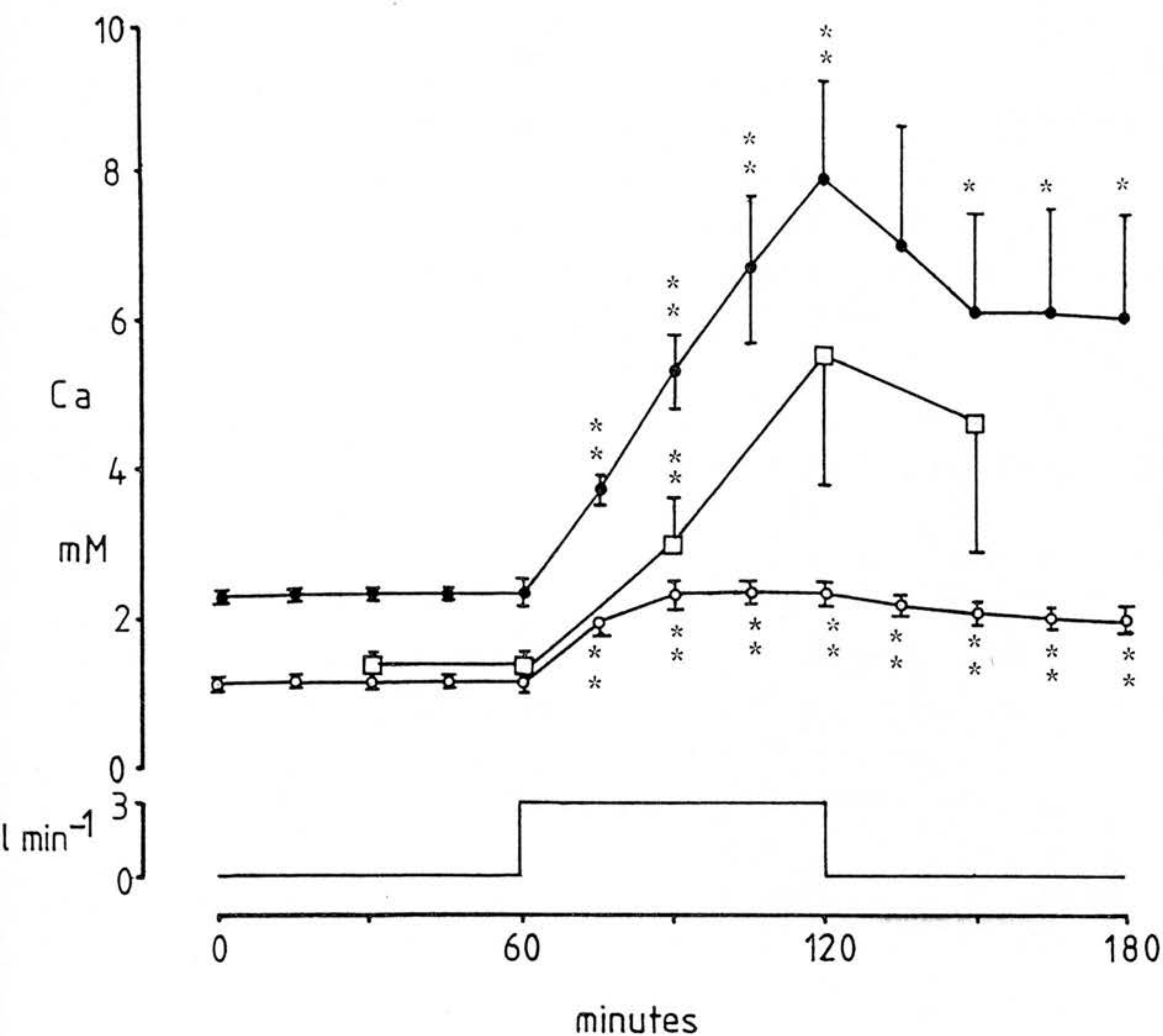


Figure 5.1 The effect of infusion of calcium borogluconate (pH 7.40, 14% w/v; shown at the bottom of the figure) in five sheep on the concentration of total (closed circles), ultrafiltrable (squares) and ionized (open circles) plasma calcium. Values were compared with their pre-infusion control concentrations: *, $P<0.05$; **, $P<0.01$.

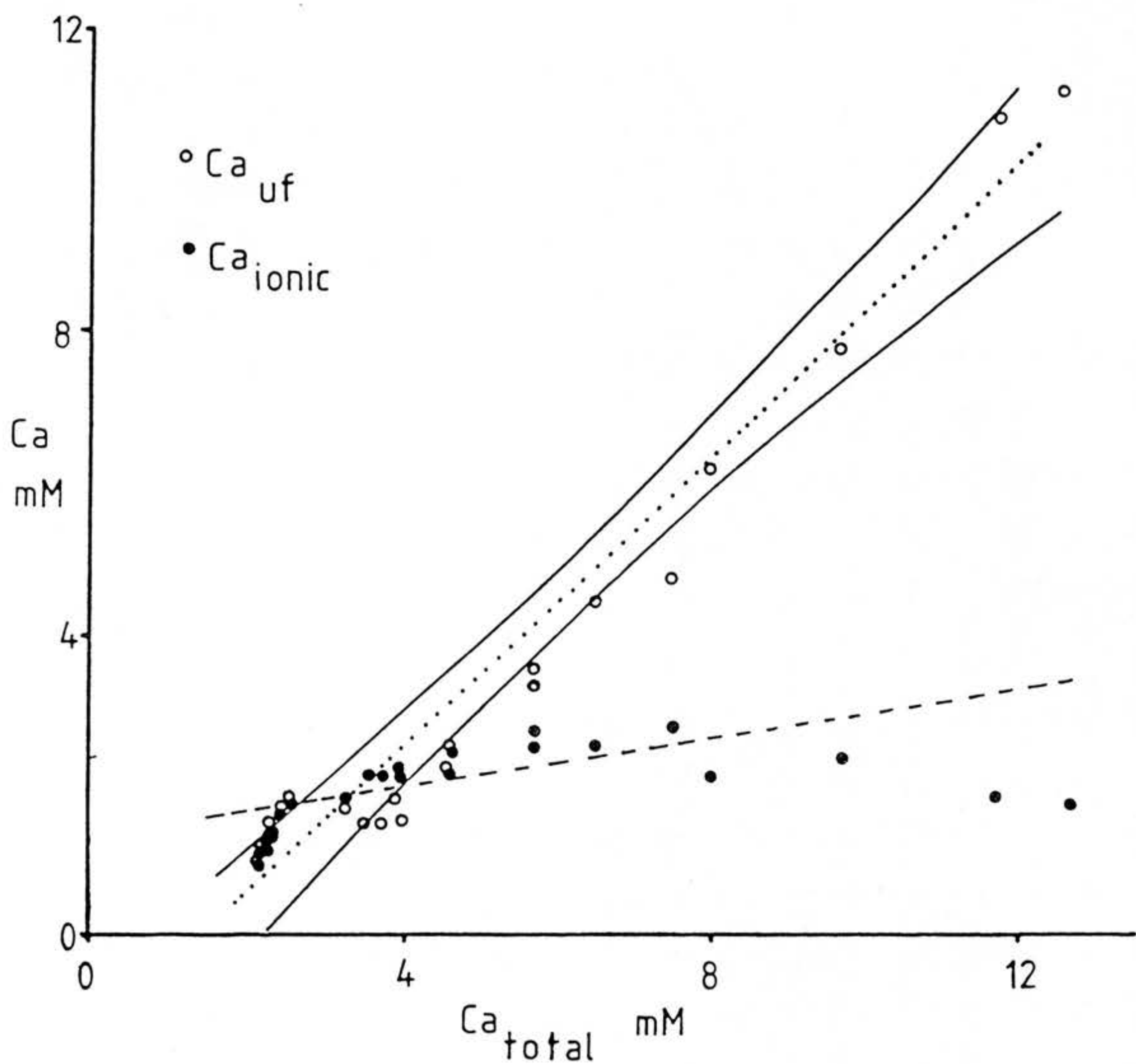


Figure 5.2 Calculated regression lines for plasma ultrafiltrable calcium (dotted line) and ionized calcium (dashed line) on total calcium during infusions of calcium borogluconate into five sheep. The ionized calcium regression line falls outside the 99% confidence intervals (curved lines) for the ultrafiltrable calcium regression ($P<0.01$).

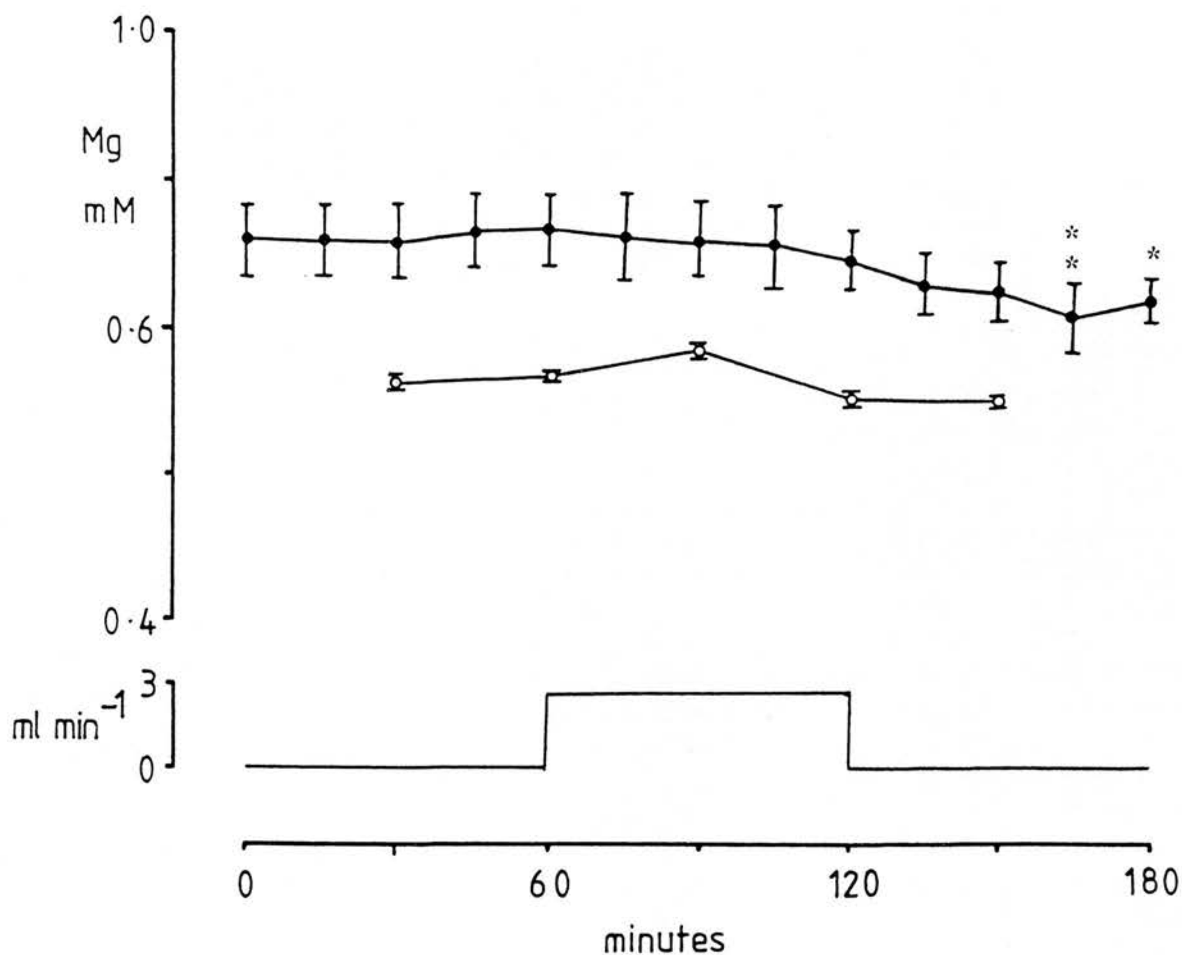


Figure 5.3 The effect of infusion of calcium borogluconate (pH 7.40, 14% w/v; shown at the bottom of the figure) in five sheep on total (closed circles) and ultrafiltrable (open circles) plasma magnesium. Values were compared with their pre-infusion control concentrations: *, P<0.05; **, P<0.02.

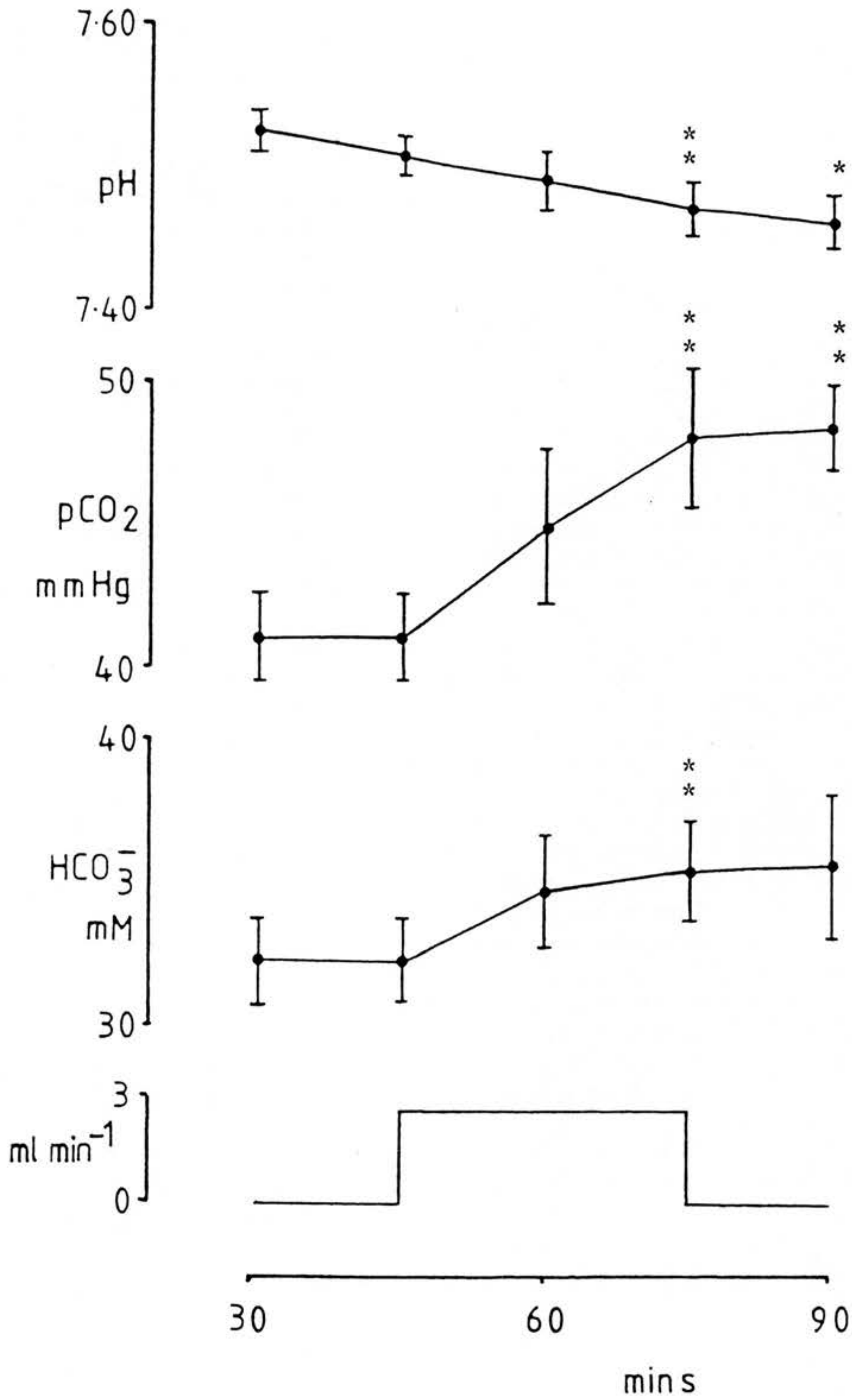


Figure 5.4 The effect of infusion of calcium borogluconate (pH 7.40, 14% w/v; shown at the bottom of the figure) in five sheep on some indices of blood acid-base status. Values were compared with their pre-infusion controls: *, P<0.03; **, P<0.01.

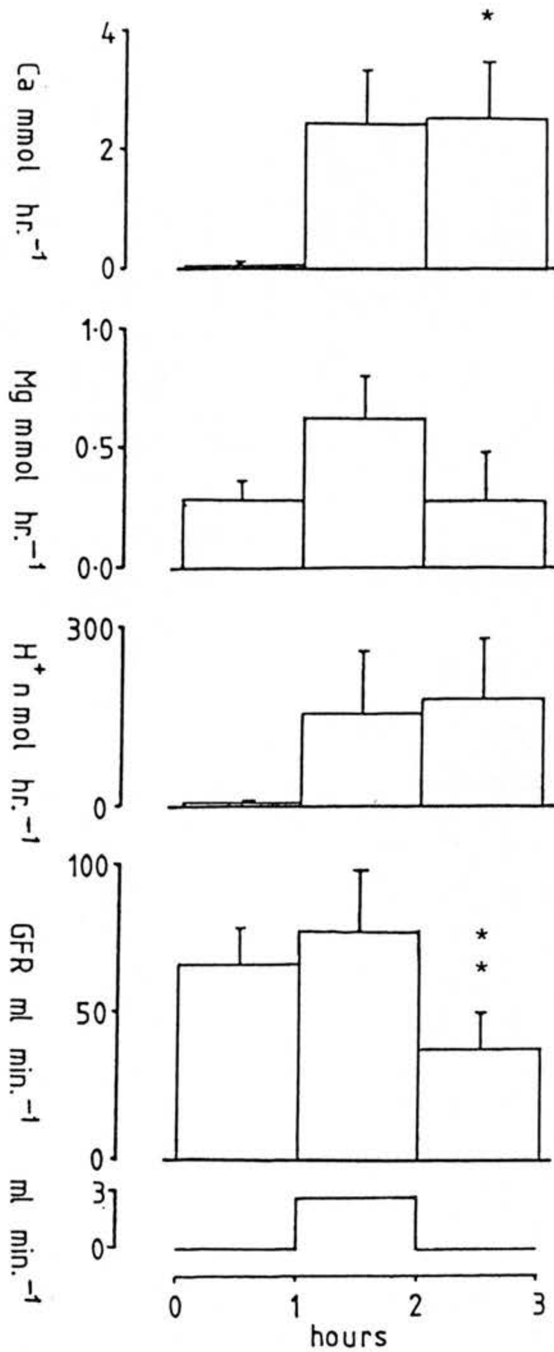


Figure 5.5 The effect of infusion of calcium borogluconate (pH 7.40, 14% w/v; shown at the bottom of the figure) in five sheep on the urinary excretion of calcium, magnesium and H^+, and the endogenous creatinine clearance. Values were compared with their control values: *, $P<0.05$; **, $P<0.02$.

4. The effect of calcium or sodium borogluconate on plasma total calcium and magnesium concentrations

Methods

Sodium borogluconate solution (20% w/v) was prepared by dissolving 16.6 g of sodium gluconate (Sigma) and 3.4 g of boric acid in water to make 100 ml. Four sheep received one of two treatments: 100 ml of sodium borogluconate (20% w/v), or 100 ml of calcium borogluconate (20% w/v) administered by subcutaneous injection, alternatively the animals were treated as controls which received no treatment. The animals were randomly assigned to these three groups, and were subjected to all three procedures at 48 hour intervals. Two hours following an injection blood sampling commenced. Four venous blood samples were taken at 90 minute intervals. Total plasma calcium and magnesium concentrations were measured.

Results

Little change in plasma calcium and magnesium occurred after the first two hours following calcium administration and so the mean for each treatment in each animal was calculated to improve the precision of the estimation. As expected, calcium borogluconate injection increased total plasma calcium concentration compared with the control period ($P<0.0005$; table 5.3). Sodium borogluconate caused plasma calcium to decrease ($P<0.015$). Neither solution had significant effects on plasma magnesium concentration, although a small decrease in plasma magnesium was seen in each case after calcium borogluconate ($P<0.1$).

Table 5.3

The effect of subcutaneous calcium or sodium borogluconates on plasma calcium and magnesium concentration in sheep

Treatment	Mg mM	Ca mM
Control	0.798 ± 0.053	2.28 ± 0.051
Sodium borogluconate (100 ml 20% w/v)	0.840 ± 0.067	*2.02 ± 0.056
Calcium borogluconate (100 ml 20% w/v)	0.621 ± 0.080	**3.59 ± 0.018

Values are means ± standard errors for four sheep. The treatments produced significant changes in calcium concentrations compared with controls; *, $P<0.015$; **, $P<0.0005$.

Discussion

In experiment 1, changes in ionized calcium were measured after the addition of either calcium borogluconate or calcium chloride to blood. In order to make such measurements valid, pH changes induced by these solutions must be considered. In this experiment the dilution effect was minimised by the use of concentrated calcium solutions. However these solutions were, of necessity, acidic since they precipitated at pH 7.40. Calcium borogluconate was associated with lower pH and lower ionized calcium than was calcium chloride. Since calcium complexes are more dissociated at low pH this difference would have the opposite effect on ionized plasma calcium to that observed. Thus it cannot account for the different ionization of the two solutions. The observed differences are most readily accounted for by the formation of calcium borogluconate complexes.

In experiment 2, the effects on ionized calcium of gluconate and/or borate were compared. The small decrease in ionized calcium produced by addition of either sodium gluconate or sodium borate was negligible compared with the effect of the two ions together. Careful pH control was instituted during this experiment so pH has no bearing on the result. The observed synergism between borate and gluconate was probably due to the formation of complexes which involve calcium, gluconate and borate.

During the intravenous infusion of calcium borogluconate the increase in total plasma calcium was primarily due to a massive increase in the non-ionized ultrafiltrable fraction of plasma calcium. It is very unlikely that an effect of this magnitude would be due to an increase in the normal calcium complexing substances

present in plasma. The effect is almost certainly due to the presence of calcium-borate-gluconate complexes.

During the infusions several effects occurred which are likely to affect calcium excretion: hypercalcaemia, an increase in plasma calcium-chelate concentration, acidosis and a reduction in GFR. In this experiment plasma ultrafiltrable calcium concentration was raised 2-3 fold and so the filtered load of calcium was greatly increased. This would cause hypercalciuria. Since a large part of the increased filtered calcium load was in the form of non-ionized complexes, tubular reabsorption of filtered calcium would have been greatly depressed, so enhancing calcium excretion. These two effects almost certainly account for the bulk of the observed increase in calcium excretion.

Additionally, acidosis promotes the excretion of calcium in the sheep (Stacy, 1969; Stacy et al, 1970) although any effect produced in this way was probably relatively insignificant. During the post infusion period GFR, as measured by the endogenous creatinine clearance, was significantly reduced. This effect has been clearly demonstrated in the dog (Edwards, Sutton and Dirks, 1974; Lins, 1979) and may result from increased renal vascular resistance (Frohlich, Scott and Haddy, 1962). Though decreased GFR might be expected to decrease calcium excretion, it seems to have been of little significance during this experiment.

Since borogluconate complexes calcium so strongly, by promoting the renal excretion of calcium it might cause hypocalcaemia. In accordance with this expectation, sheep given sodium borogluconate by subcutaneous injection showed significant reductions in total plasma calcium.

Following the intravenous infusion of calcium borogluconate in

sheep, alterations in urinary pH, calcium excretion and GFR did not reach significance until the post infusion period. This was probably a result of dead space in the urinary bladder and collection apparatus, since the extremely variable urine flow rate and the volume of the catheter and tubing would account for delays in collection of approximately 5-40 minutes.

Intravenous infusion of calcium borogluconate in sheep was followed by a decrease in plasma magnesium. Though the increase in magnesium excretion observed during these infusions was not significant ($P<0.09$), hypermagnesuria and hypomagnesaemia may result from an increase in filtered calcium load causing competitive depression of the renal tubular reabsorption of magnesium (Samiy et al, 1960; Massry et al, 1969; Quamme et al, 1980). However since complexing agents which complex calcium almost invariably also complex magnesium, hypomagnesaemia may also result from the formation and elimination of magnesium-borogluconate complexes. Although clear evidence supporting this suggestion is lacking, the possibility merits further investigation.

If the hypomagnesaemia associated with calcium borogluconate infusion was the result of calcium and magnesium competing for renal tubular reabsorption, it seems likely that the administration of magnesium salts might be followed by hypocalcaemia. Various studies have demonstrated this effect (Kelly et al, 1960; Keměny et al, 1961; Pors Nielsen, 1970). The possibility that sheep might also show this effect was briefly investigated. Four animals were used in a cross over experiment, the experimental protocol and results are shown in figure 5.6. Administration of magnesium chloride to sheep was found to cause hypermagnesaemia ($P<0.005$)

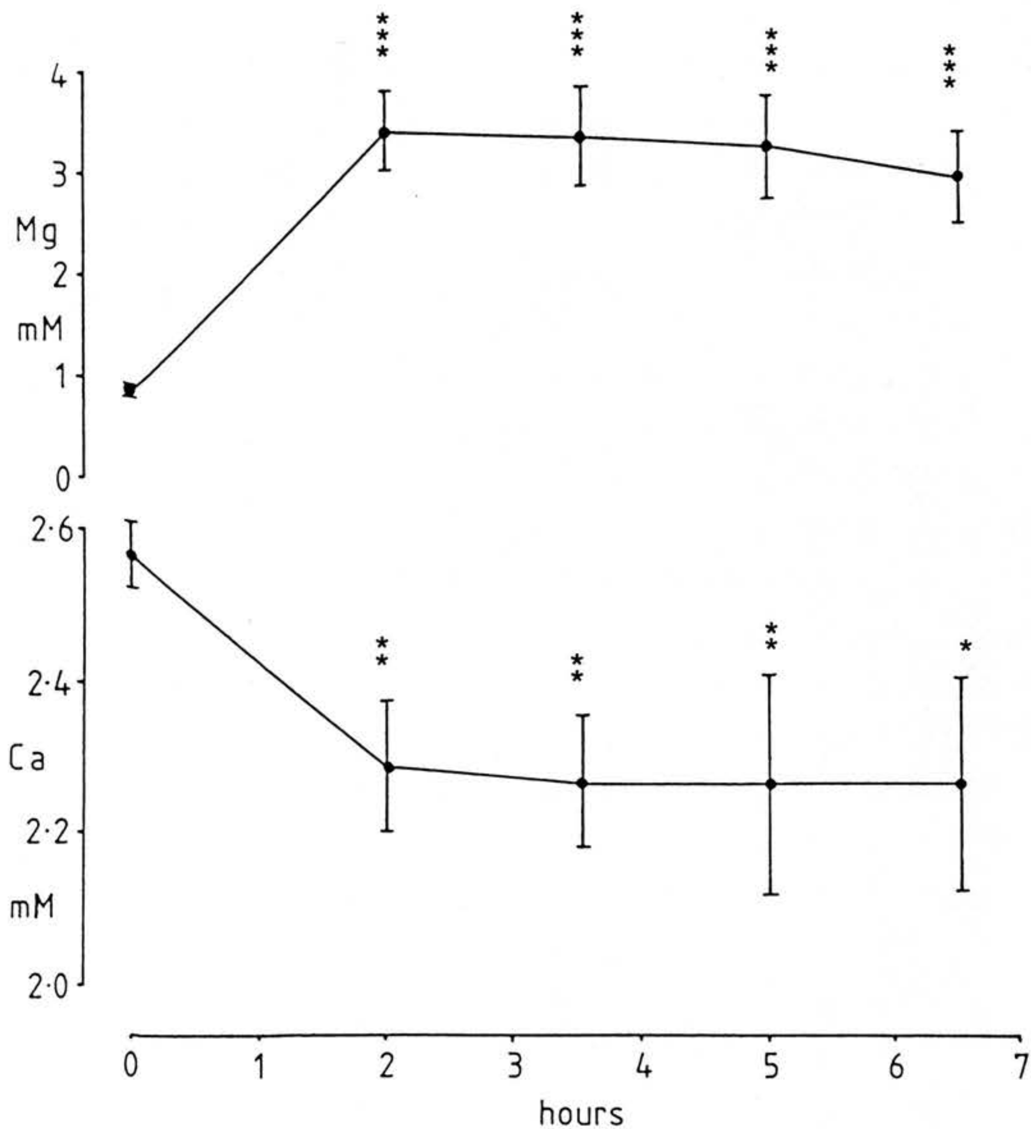


Figure 5.6 The effect of administering magnesium chloride on total plasma calcium and magnesium concentrations in four sheep. At time 0.0 hrs. 150 ml of solution (pH 7.40, 10% w/v) was administered by subcutaneous injection. Plasma concentrations were compared with their pre-administration levels: *, P,0.02; **, P<0.01, ***, P<0.005.

associated with hypocalcaemia ($P<0.01$). Clearly the result supports this hypothesis.

In this chapter evidence is presented showing that borogluconate is a powerful calcium complexing agent *in vitro* and *in vivo*. Though the scope of this work does not extend to a clinical appraisal of calcium borogluconate, these findings suggest that the clinical efficacy of calcium borogluconate is likely to differ from that of more completely ionized calcium salts. This study suggests that clinical comparisons of calcium borogluconate with more completely ionized salts might prove a fruitful area for further research.

CHAPTER VI

THE BINDING OF CALCIUM AND MAGNESIUM BY ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP) IN VITRO AND IN VIVO. ITS EFFECT ON PLASMA AND URINARY CALCIUM AND MAGNESIUM IN THE SHEEP

Summary

1. Calcium and magnesium binding by EHDP was studied *in vitro* using divalent cation selective electrodes and ultrafiltration. EHDP was then infused intravenously into sheep. Blood was collected every half hour and urine for periods of one hour, before, during and after the infusion. Total, ultrafiltrable and ionized calcium, and total and ultrafiltrable magnesium concentrations were determined in plasma. Endogenous creatinine clearance and the urinary excretion of calcium, magnesium, sodium, potassium and hydrogen ion were measured.

2. *In vitro* EHDP added to calcium or magnesium containing solutions caused a decrease in ultrafiltrable and ionized calcium and magnesium concentrations. The decrement in the ultrafiltrable fraction was very similar in both plasma and saline of similar ionic composition.

3. Addition of EHDP to blood *in vitro* also depressed ionized

calcium concentration, the effect was similar to the effect of pyrophosphate but greater than the effect of EDTA.

4. These studies showed that *in vitro* EHDP complexed both calcium and magnesium. The decrease in plasma ultrafiltrable calcium and magnesium seemed to be the result of agregation of complexes and not the result of protein binding of EHDP-divalent cation complexes.

5. Intravenous infusions of EHDP into sheep resulted in:

(i) increased total plasma calcium concentrations;

(ii) decreased ultrafiltrable plasma calcium and magnesium concentrations;

(iii) decreased ionized plasma calcium concentration;

(iv) increased urinary calcium excretion which was primarily due to an increase in the non-ultrafiltrable fraction and was not associated with a change in endogenous creatinine clearance;

(v) increased urinary non-ultrafiltrable magnesium excretion whilst total excretion did not change significantly;

(vi) increased renal excretion of hydrogen ions.

6. These observations indicate calcium and magnesium complex formation *in vitro* and *in vivo*. Since the complexes formed were only partially ultrafiltrable, and since EHDP infusion resulted in hypercalcaemia, it seems likely that the clearance of EHDP is less than other ligands, such as EDTA, whose clearance is equal to GFR.

Introduction

Several studies have been carried out which show that diphosphonates complex calcium (Carroll and Irani, 1968; Grabenstetter and Cilley, 1971). Large aggregates were produced, the maximum overall EHDP:calcium binding ratio was found to be 1:1·75. Wiers (1971) demonstrated the formation of large complexes of the order of 10^4 Daltons and with approximate radii of 2.6 nm. However these studies were carried out at pH 11 and at non physiological calcium concentrations. The extent to which these results can be extrapolated to studies *in vivo* is not clear.

Bisaz *et al* (1978) showed that ^{32}P-pyrophosphate and various diphosphonates, including ^{14}C-EHDP, were partly non-ultrafiltrable in plasma. It was not clear whether this effect was due to protein binding, or the formation of large complexes.

Complex formation by EHDP was examined in plasma and in artificial solutions. EHDP infusions were then made in sheep to examine binding *in vivo.*

Methods and Results

1. The ionization of calcium and magnesium in solutions containing EHDP

Methods

Aqueous solutions containing 15 mM tris-HCl (pH 7.40), and 21.2 or 39.6 micro-mol EHDP (donated by Proctor and Gamble Ltd) in 40 ml were titrated with 0.80 M $MgCl_2$ or 0.80 M $CaCl_2$ in 15 mM tris. This was carried out in a 50 ml beaker on a magnetic stirrer. Ionic divalent cation activities were measured using the Orion divalent ion electrode.

Intersections of the asymptotes of titration curves (such as those in figures 6.1 and 6.2) with the abscissa indicates the theoretical maximum amount of cation bound by each amount of EHDP. Since in the present investigation EHDP was never saturated by divalent cation, rather than attempting to extrapolate the data to indicate theoretical maxima, lines of gradient 1.0 were drawn to indicate the maximum cation bound during each titrations. Thus the values indicated by such a manouvre in fact represent binding ratios which are likely to be less than the theoretical maxima.

Results

The maximum volume of calcium or magnesium added to the initial volume (40 ml) was 0.12 ml. Figure 6.1 shows that at the start of the titration nearly all added calcium was not ionized whereas at the end, increments in total and ionized calcium were almost equal. Doubling the concentration of EHDP had the effect of shifting the inflexion of the curve to the right, thus the maximum amount of

calcium bound was increased. Magnesium showed very similar effects (figure 6.2). At the maximum titres of magnesium or calcium, 44.3 micro-mol calcium or 36.9 micro-mol of magnesium were non-ionized in the presence of 21.2 micro-mol EHDP. With 39.6 micro-mol EHDP, 72.5 micro-mol calcium, or 65.7 micro-mol of magnesium were non-ionized. These figures represent binding ratios of 1.7-2.1. The Scatchard Plot can be used to obtain values for binding ratios.When the data are plotted in this way the points fall on a straight line.The binding ratios were 2.26 for calcium and 1.83 for magnesium.

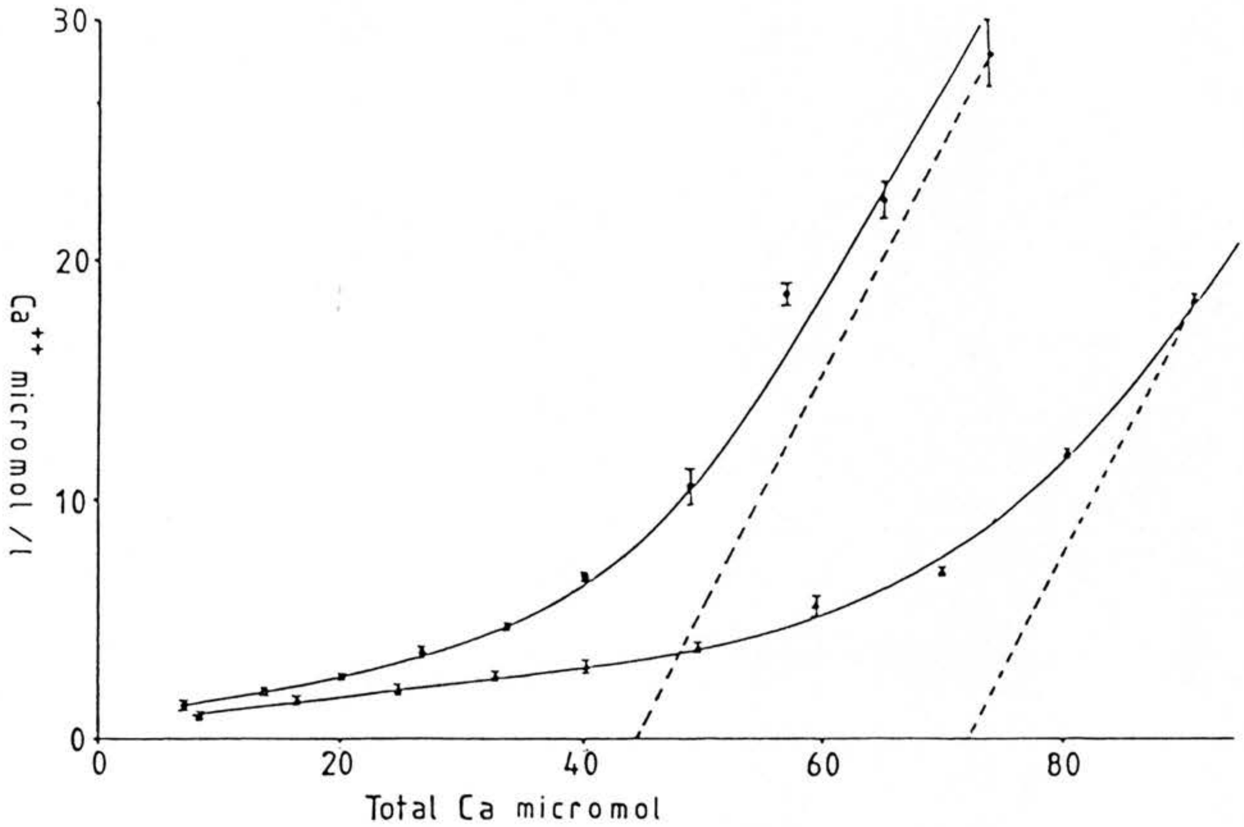


Figure 6.1 Active calcium, measured by the divalent cation electrode, during titration of 21.2 micro-mol (closed circles) or 39.6 micro-mol (triangles) EHDP in 40 ml of tris buffer, with 0.80 M calcium chloride. The intersection of the dashed lines (of gradient 1.0) and the x-axis indicates the maximum calcium bound by each amount of EHDP. Each point is a mean and standard error of four observations.

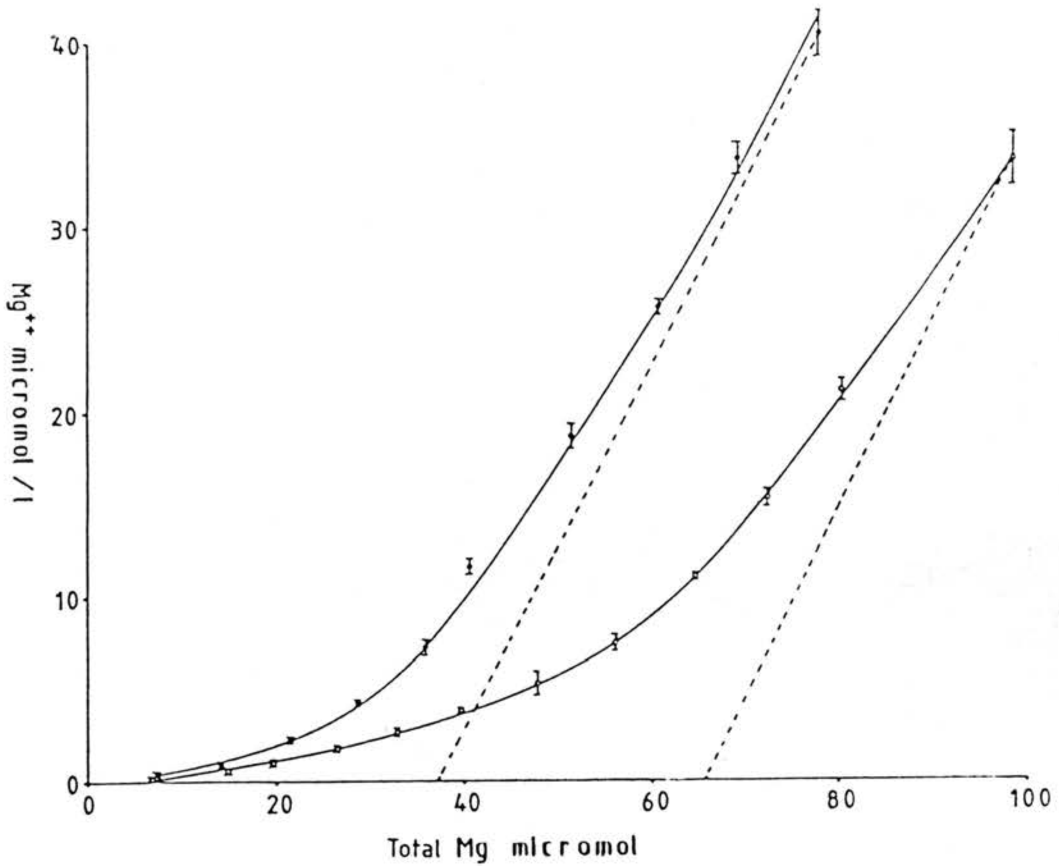


Figure 6.2 Active magnesium, measured by the divalent cation electrode, during titration of 21.2 micro-mol (closed circles) or 39.6 micro-mol (open circles) EHDP in 40 ml of tris buffer, with 0.80 M magnesium chloride. The intersection of the dashed lines (of gradient 1.0) and the x-axis indicates the maximum amount of magnesium bound by each amount of EHDP. Each point is a mean and standard error of four observations.

2. Ultrafiltrable calcium and magnesium in solutions containing EHDP

Methods

Solutions were prepared containing 150 mM NaCl, 0.53 mM EHDP, 4.0 mM KCl, 15 mM tris-HCl (pH 7.40) and either $MgCl_2$ or $CaCl_2$ at 0-2 mM. These solutions were ultrafiltered and total magnesium or calcium concentrations measured in ultrafiltrates.

Results

Figure 6.3 shows that only part of the calcium and magnesium in the solutions was ultrafiltrable ($P<0.001$). Over the studied EHDP concentration ranges, the proportion of the total calcium and magnesium which was ultrafiltrable was remarkably constant ($47.3 \pm 2.3\%$ $n = 7$) and ($81.7 \pm 0.9\%$ $n = 7$) respectively.

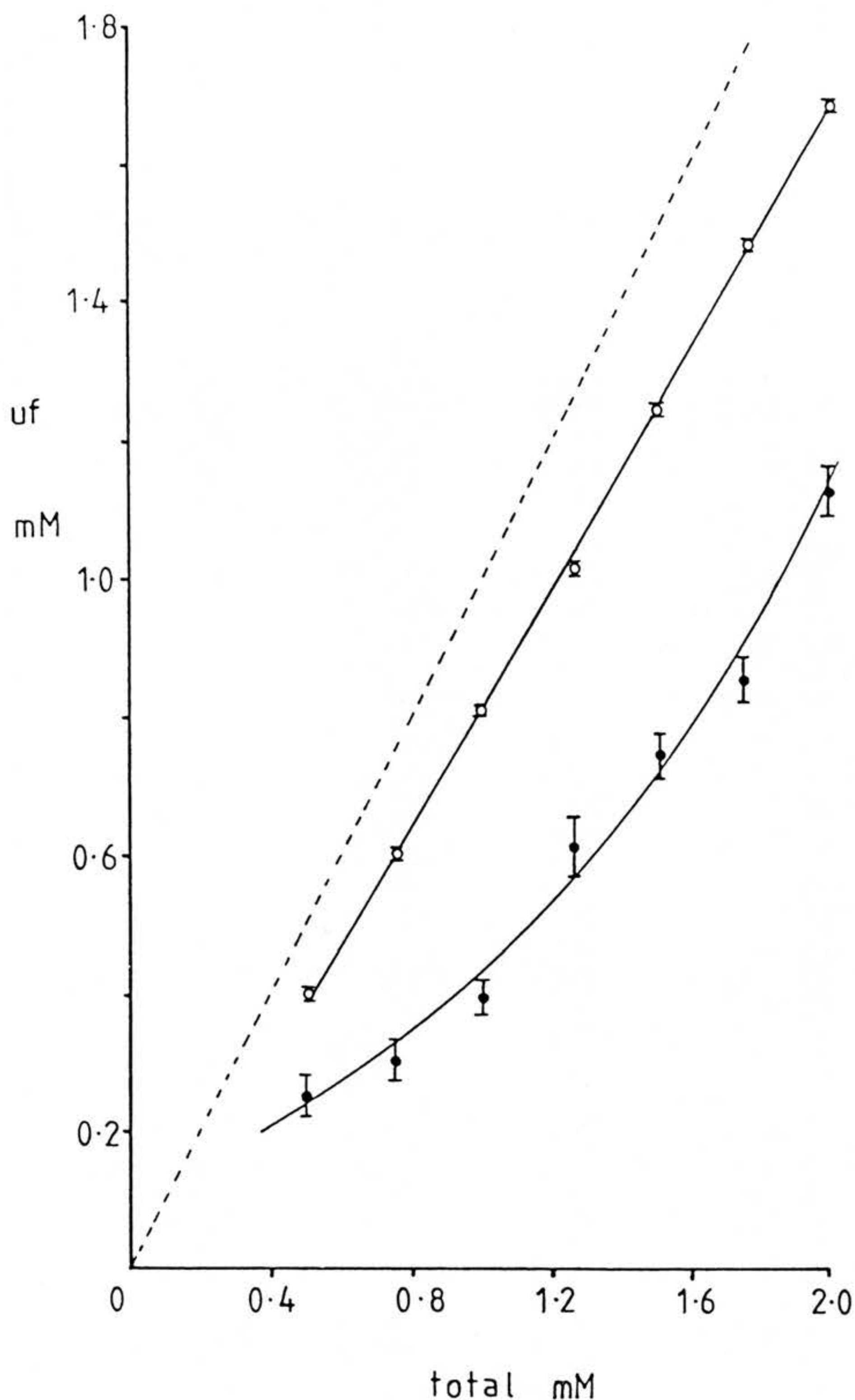


Figure 6.3 Ultrafiltrable calcium (closed circles) or magnesium (open circles) in solutions containing 0.53 mM EHDP and varying concentrations of either calcium or magnesium. The expected line, if ultrafiltrable and total concentrations were equal, is shown dashed. Each point is a mean and standard error of four observations.

3. Comparison of the ultrafiltrable calcium and magnesium fractions in plasma and in solutions

Methods

Portions (12, 24 or 36 micro-l) of 160 mM EHDP solutions were put in 5 ml syringes which were then filled to the mark with heparinised sheep plasma. The final EHDP concentrations were 0.38, 0.77 and 1.15 mM. The plasma was ultrafiltered and calcium and magnesium concentrations in ultrafiltrates measured as described in Chapter II. Solutions were then made up containing 150 mM NaCl, 4.0 mM KCl, 15 mM tris-HCl (pH 7.40) and calcium and magnesium at concentrations equal to those previously determined in plasma ultrafiltrates. These solutions were ultrafiltered in triplicate.

Results

In this experiment the maximum dilution produced by the addition of EHDP to plasma was 0.7%. The solutions for comparison with plasma contained 1.53 mM $CaCl_2$ and 0.690 mM $MgCl_2$. Figure 6.4 shows that at every EHDP concentration, ultrafiltrable calcium concentration and was the same in plasma as in artificial solutions; similarly for magnesium.

The ultrafiltrable magnesium concentration in plasma containing EHDP, at 0.77 and 1.15 mM, was lower than in plasma containing no EHDP ($P<0.01$). The effect of EHDP on ultrafiltrable calcium was larger, significant decreases were observed at all three concentrations (0.38 mM EHDP, $P<0.02$; 0.77 mM EHDP, $P<0.01$; 1.15 mM EHDP, $P<0.001$).

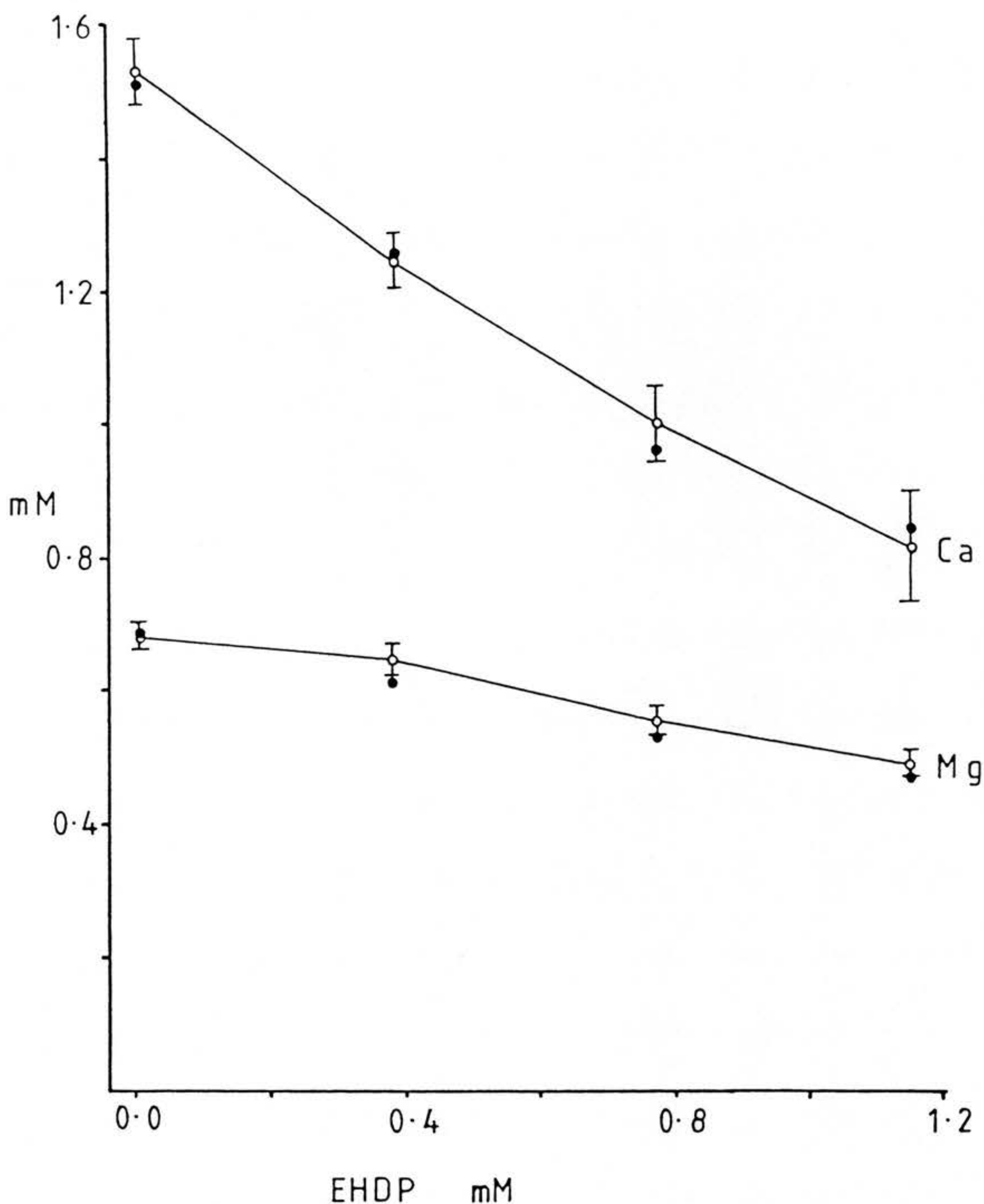


Figure 6.4 The effect of EHDP concentration on plasma ultrafiltrable calcium and magnesium (open circles) compared with the effect of EHDP concentration on ultrafiltrable calcium and magnesium in saline solutions of similar ionic composition (closed circles). Each point is a mean and standard error of four observations.

4. The reduction in ionized and ultrafiltrable plasma calcium concentrations after the addition of EHDP

Methods

Portions (12, 24 or 36 micro-l) of 160 mM EHDP solutions were put in 5ml syringes, which were then filled to the mark with heparinised sheep plasma. Ionized calcium was determined with the Nova-2 analyser and the remaining plasma ultrafiltered in duplicate. Calcium and magnesium were measured in the ultrafiltrates.

Results

Addition of EHDP to plasma (table 6.1) caused a reduction in ultrafiltrable and ionized calcium at all three EHDP concentrations. Approximately 70-80% of the non-ionized calcium was also non-ultrafiltrable. The decrements in ionized and ultrafiltrable calcium only differed significantly at the highest EHDP concentration (1.15 mM) ($P<0.05$).

Table 6.1

Comparison of the reduction in ultrafiltrable and ionized calcium produced by the addition of EHDP to plasma

Final [EHDP] mM	Reduction in ultrafiltrable calcium mM	Reduction in ionized calcium mM	
0.38	0.27 ± 0.03 (3)	0.40 ± 0.08 (3)	ns
0.77	0.46 ± 0.08 (3)	0.58 ± 0.01 (3)	ns
1.15	0.66 ± 0.05 (3)	0.80 ± 0.02 (3)	$P<0.05$

The figures are mean ± standard error (number of observations). Comparisons were made between the ultrafiltrable and ionized fractions at each EHDP concentration. The ultrafiltrable and ionized calcium concentrations in untreated plasma were 1.53 mM and 1.19 mM respectively.

5. The effect of EHDP, pyrophosphate and EDTA on ionized calcium in plasma

Methods

Syringes (2 ml) containing aliquots of 0, 0.01, 0.02, to 0.1 ml of 60 mM EHDP were filled to the 2 ml mark with heparinised sheep plasma, ionized calcium was then measured. On separate occasions the experiment was repeated using either 60 mM tetrasodium pyrophosphate, or 60 mM EDTA.

Results

The effects of EHDP and pyrophosphate on ionized calcium (figure 6.5) were similar. EDTA complexes calcium in the ratio 1:1 and had a significantly smaller effect on ionized calcium at the lower concentrations, but a greater effect at the highest concentrations. Increasing EDTA concentration was associated with an almost linear fall in ionized calcium, whereas EHDP and pyrophosphate showed distinctly non-linear relationships with ionized calcium.

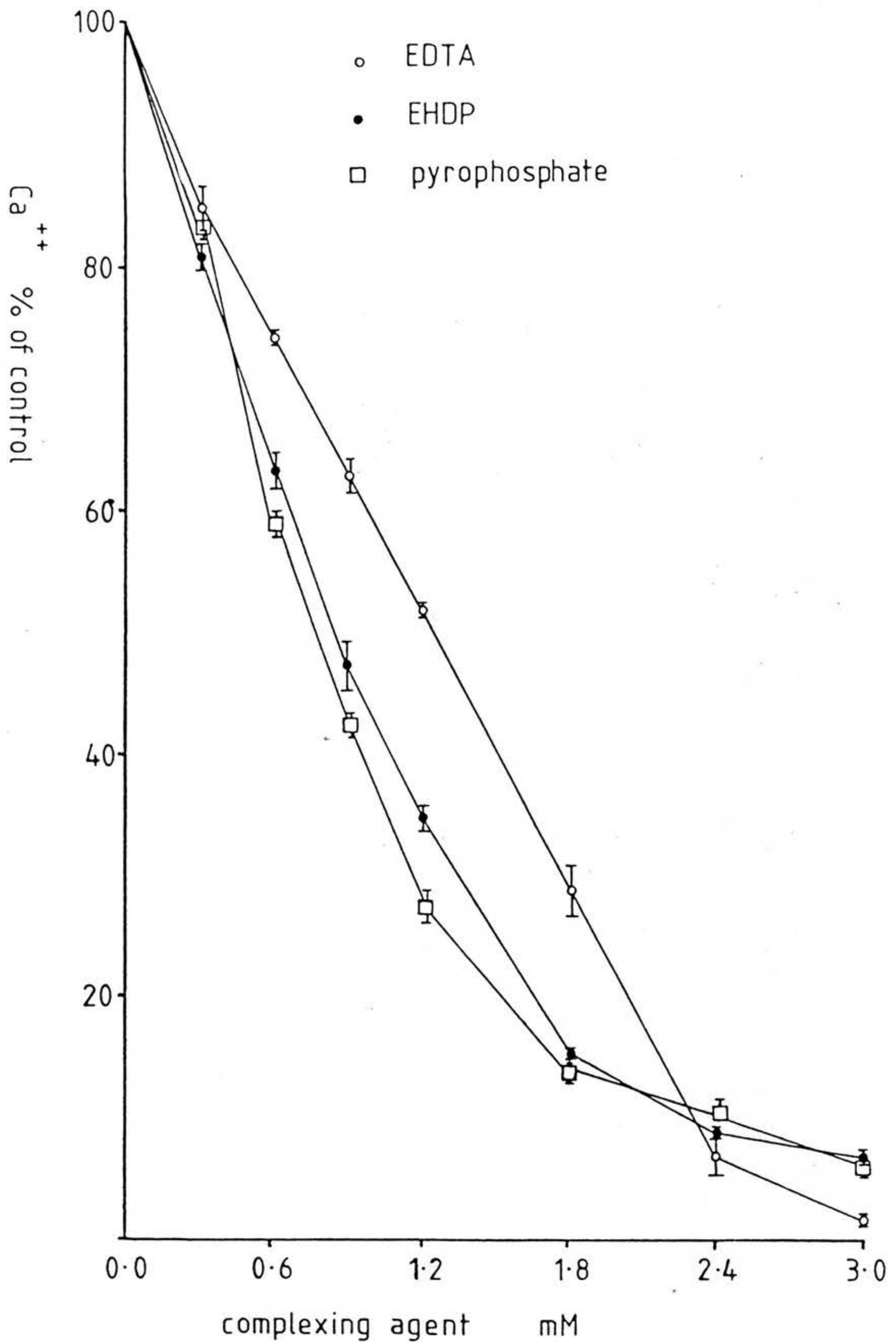


Figure 6.5 The effect of some ligands on ionized calcium in plasma. Each point is a mean and standard error of four observations. Untreated plasma contained 1.20-1.22 mM calcium.

6. The effect of EHDP infusions in sheep on urinary and plasma calcium and magnesium fractions

Methods

Four sheep were equipped with urinary and jugular venous catheters. The pH of EHDP (4% w/v) was adjusted to 7.40 with sodium hydroxide, this gave an approximately isotonic solution. Intravenous infusion of sodium chloride solution (0.9% w/v) was made during two hour control periods before and after EHDP infusion. EHDP was infused at a priming dose of 2.5 micro-mol $min^{-1}kg^{-1}$ for half an hour followed by a maintenance dose of 0.7 micro-mol $min^{-1}kg^{-1}$ for two hours. EHDP solution was diluted as required with saline so as to maintain a constant infusion rate of 1.2 ml min^{-1}. Blood samples were collected every 30 minutes, urine was collected for 60 minute periods except for the 30 minute period during administration of the priming EHDP dose.

Results

During EHDP infusion, total plasma calcium concentration increased (figure 6.6; $P<0.01$) whilst ionized calcium decreased ($P<0.02$). As a % of the total, plasma ultrafiltrable calcium decreased ($P<0.05$). Total plasma magnesium concentration did not change significantly (figure 6.7), but was significantly correlated with total calcium ($r=0.667$, $P<0.001$). Ultrafiltrable magnesium concentration (as % of total) decreased ($P<0.05$).

Total urinary calcium excretion increased ($P<0.01$; figure 6.8). The increment was entirely due to the non-ultrafiltrable fraction ($P<0.01$), since the ultrafiltrable fraction did not change significantly. The excretion of non-ultrafiltrable urinary

magnesium also increased ($P<0.001$). In this case the increment was a result of a combination of a non-significant increase in total, and a non-significant decrease in ultrafiltrable magnesium excretion. GFR, as measured by endogenous creatinine clearance did not change, and neither did sodium or potassium excretion (table 6.2). Hydrogen ion excretion increased ($P<0.05$) during the infusion.

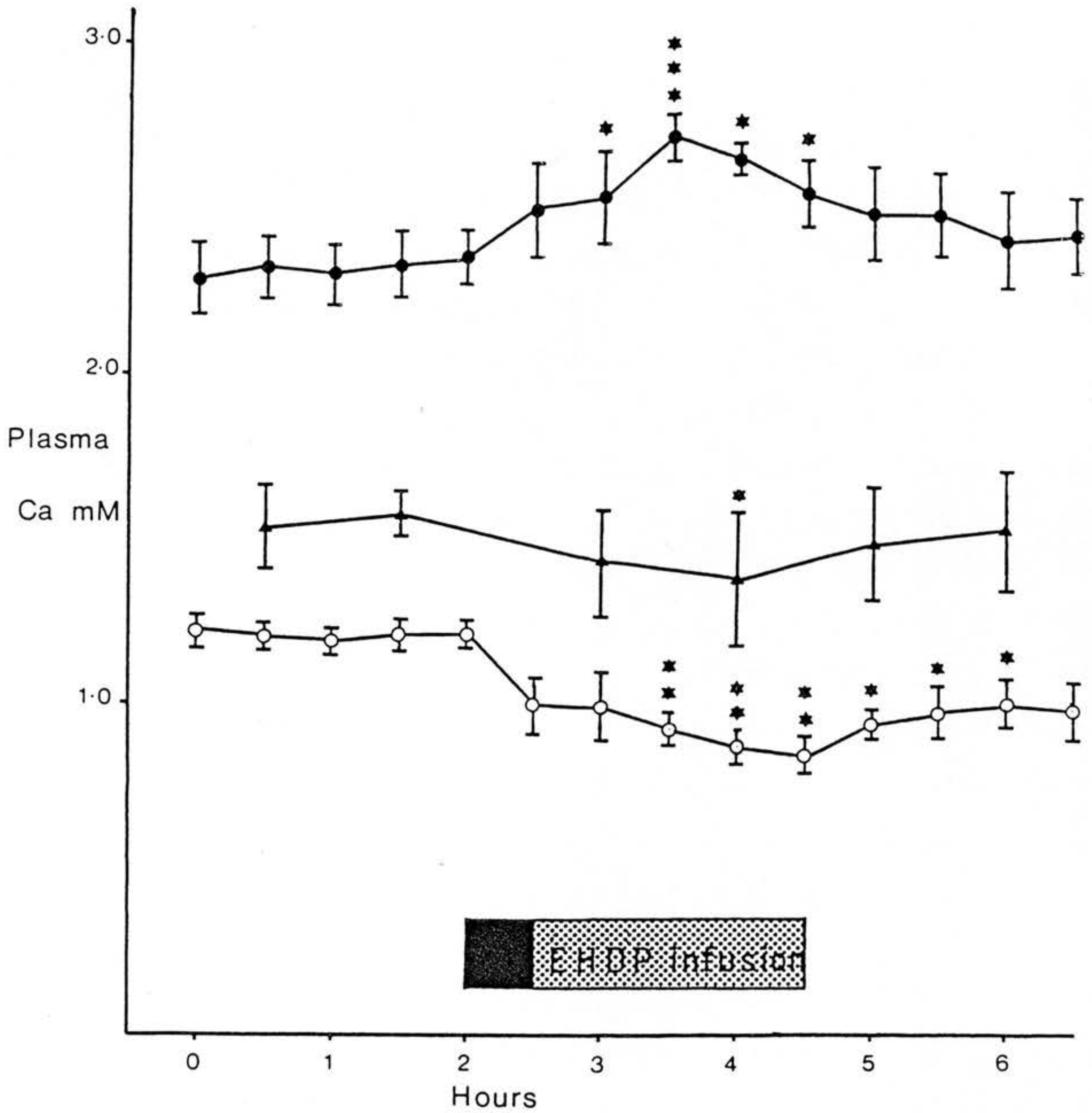


Figure 6.6 The effect of EHDP infusions in four sheep on the concentration of total (closed circles), ultrafiltrable (triangles) and ionized (open circles) plasma calcium. EHDP was infused at 7 micro-mol $min^{-1}kg^{-1}$ for 120 minutes (shown dotted), following a 30 minute priming infusion of 25 micro-mol $min^{-1}kg^{-1}$. Values were compared with their pre-infusion controls: *,P<0.05; **, P<0.02; ***, P<0.01.

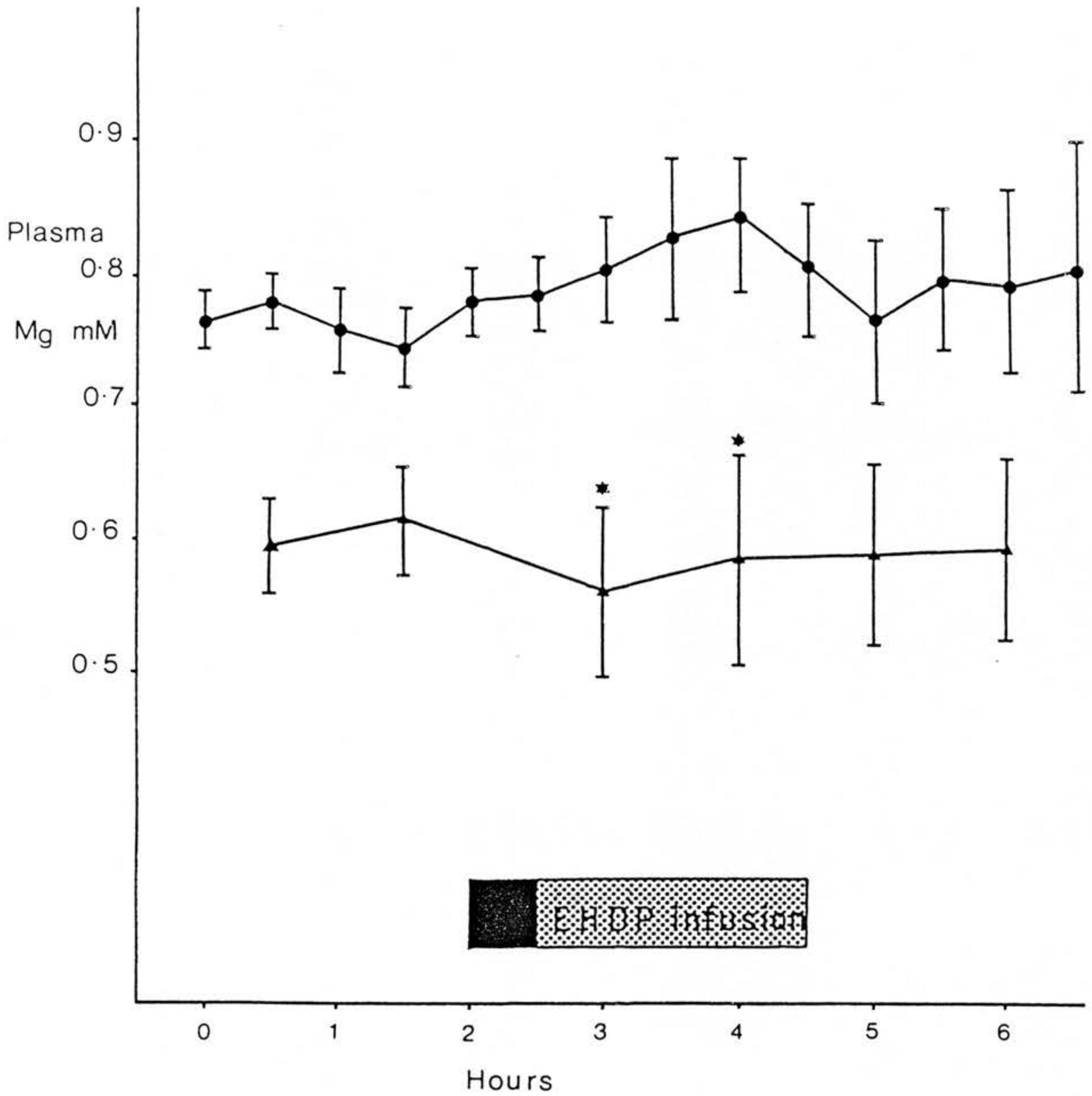


Figure 6.7 The effect of EHDP infusions in four sheep on the concentration of total (closed circles) and ultrafiltrable (triangles) plasma magnesium. EHDP was infused at 7 micro-mol $min^{-1}Kg^{-1}$ for 120 minutes (shown dotted), following a 30 minute priming infusion of 25 micro-mol $min^{-1}Kg^{-1}$. Values were compared with their pre-infusion controls: *,P<0.05.

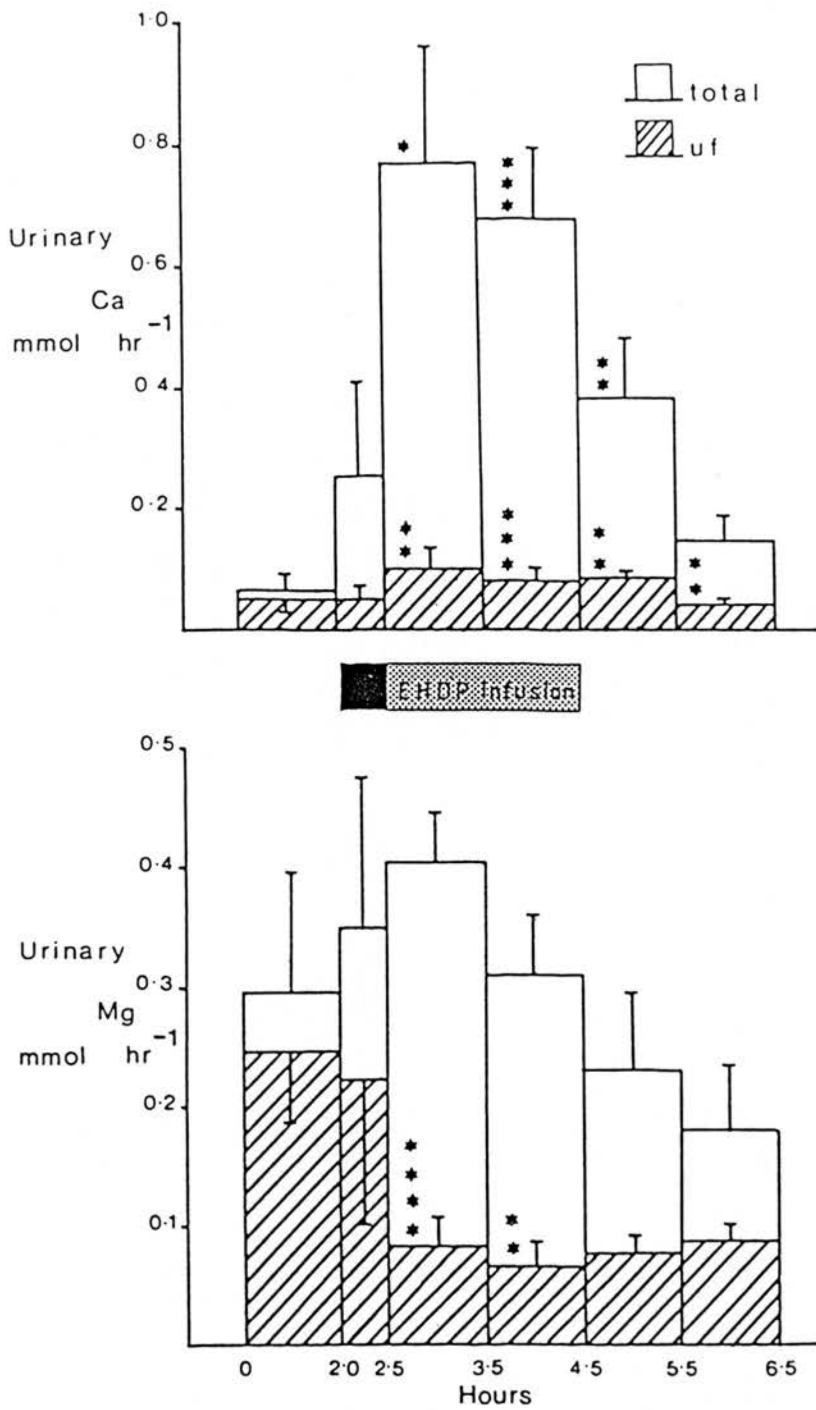


Figure 6.8 The effect of EHDP infusions in four sheep on the hourly excretion of total and ultrafiltrable calcium and magnesium. EHDP was infused at 7 micro-mol $min^{-1}Kg^{-1}$ for 120 minutes (shown dotted), following a 30 minute priming infusion of 25 micro-mol $min^{-1}Kg^{-1}$. Total and non-ultrafiltrable values were compared with their pre-infusion controls: *,P<0.05; **, P<0.02; ***, P<0.01; ****,P<0.001.

Table 6.2

The effect of EHDP infusion in the sheep on the excretion of sodium, potassium and H^+, and GFR

Collection period hours	Sodium mmol hr^{-1}	Potassium mmol hr^{-1}	H^+ nmol hr^{-1}	GFR ml min^{-1}
		Control		
0-1	5.6 ± 1.9	12.2 ± 5.7	0.93 ± 0.41	93 ± 12
1-2	5.2 ± 1.6	8.0 ± 3.5	0.74 ± 0.29	86 ± 26
	EHDP infusion rate: 25 micro-mol $kg^{-1}min^{-1}$			
2-2.5	9.7 ± 3.4	9.4 ± 5.1	1.05 ± 0.33	80 ± 10
	EHDP infusion rate: 7 micro-mol $kg^{-1}min^{-1}$			
2.5-3.5	10.7 ± 3.1	10.2 ± 3.2	*5.08 ± 1.48	74 ± 6
3.5-4.5	6.0 ± 2.0	9.5 ± 1.4	3.96 ± 1.95	72 ± 15
		Post-infusion period		
4.5-5.5	2.9 ± 1.1	8.7 ± 1.9	1.81 ± 0.44	82 ± 8
5.5-6.5	3.7 ± 0.5	8.4 ± 1.9	1.86 ± 0.57	75 ± 11

Each figure is a mean ± standard error of four observations. The values were compared with the mean of their respective controls; *, $P<0.05$.

Discussion

In vitro

Evidence presented by Bisaz et al (1978) showed that EHDP in plasma was partly non-ultrafiltrable through filters with a cut off point of 50,000 Daltons. They did not show whether this was due to protein binding or the formation of large complexes.

In the present study EHDP was found to form non-ultrafiltrable non-ionized complexes with calcium and magnesium in vitro. EHDP reduced ultrafiltrable calcium and magnesium to the same extent in plasma as in protein free artificial solutions. It seemed, therefore, that the effect of EHDP on calcium and magnesium binding was the result of the formation of large non-ultrafiltrable aggregates and not protein binding of EHDP-divalent cation complexes.

The non-ultrafiltrable nature of calcium and magnesium in the presence of EHDP might have been due to adsorption of EHDP-divalent cation complexes by visking tubing. This hypothesis was rejected following hot ashing of strips of visking tubing which had been soaked in EHDP-Ca-Mg solutions. The ash calcium and magnesium content was no greater than would be expected as a result of the water content of the visking tubing.

In this study EHDP seemed to complex calcium more strongly than magnesium. This was suggested by ionic determinations in solutions; for a given titre of calcium or magnesium the former ion was more completely bound (comparison of figures 6.1 and 6.2). In ultrafiltrates prepared from aqueous solutions, or from plasma, considerably more of the calcium was non-ultrafiltrable than was

magnesium. However despite the apparently greater affinity of EHDP for calcium, magnesium binding occurred to a significant extent both in vitro and during EHDP infusions.

Since EDTA complexes calcium stoichiometrically it was used in experiment 5 to sequester specific amounts of plasma calcium. This indicated the reduction in plasma calcium associated with chelation of specific amounts of plasma calcium, in the presence of the buffering ability of plasma proteins. Thus figure 6.5 shows that the calcium binding ratios of EHDP and pyrophosphate were both greater than 1:1 (the ratio for EDTA). Figures 6.1 and 6.2 also indicate that in buffered solutions the amount of non-ionized calcium or magnesium reached maxima approaching twice the amount of EHDP present. This suggested maximum binding ratios similar to the ratio of 1.75 proposed by Grabenstetter and Cilley (1971).

The similarity between calcium binding by pyrophosphate and EHDP in whole blood suggests that pyrophosphate, like EHDP, forms large aggregates. Little evidence on this point exists in the literature although Bisaz et al (1978) showed that calcium-^{99m}Tc-pyrophosphate was not completely ultrafiltrable. This might have been due to the formation of large aggregates.

Although EHDP clearly formed large, non-ultrafiltrable calcium and magnesium complexes in plasma, it was not certain whether or not these complexes would be filtered by the glomerulus. The pore radius of visking tubing is 1.3-1.9 nm (Renkin, 1954; Durbin, 1960), slightly smaller than the estimated radius of the calcium-EHDP aggregates (2.6 nm; Wiers, 1971). The glomerular pore size has not been estimated in the sheep, however in most species molecules larger than about 70,000 Daltons are not filtered. Thus although

the complexes may be too large to be readily ultrafiltered by visking tubing, their molecular weight (of the order of 10,000 Daltons) would suggest that they probably pass through the glomerulus.

Such large particles almost certainly flow less readily through the glomerular membrane than smaller ones. This phenomenon is well recognised, and has been used to estimate the pore size of artificial membranes. Particles undergoing ultrafiltration are subject to resistance in proportion to their size. Dextran infusions in dogs have been used to demonstrate the relationship between filtered fraction and molecular weight over the range 10,000-70,000 Daltons (Giebisch, Lauson and Pitts 1954). In a similar way large EHDP aggregates are probably subject to considerable resistance as they pass through the glomerular filter. If large partially filtrable complexes are formed in blood then this would explain why the EHDP clearance was only 27% of the simultaneous creatinine clearance (Hughes et al, 1977).

In vivo

EHDP has been found to cause acidosis in nephrectomised rats, but not in intact animals (Goulding and Broom, 1979). It was suggested that the effect was related to inhibition of bone resorption. The increased H^+ excretion observed during EHDP infusions in sheep may have resulted from similar mechanisms, however since the present observations were made in intact animals a different mechanism may be involved.

The changes observed in plasma calcium and magnesium fractions during EHDP infusion suggest the formation of large non-

ultrafiltrable complexes which are not available for renal tubular reabsorption. Thus, hypercalciuria may have been due to glomerular filtration of some of these aggregates. The fact that EHDP infusion also brought about a large increase in the non-ultrafiltrable fractions of urinary calcium and magnesium is consistent with glomerular filtration of calcium and magnesium complexes.

Comparison of the data collected during intravenous EDTA (Chapter IV) and EHDP infusions shows differences which can be explained on the basis of the formation of poorly filtered EHDP aggregates. Though such uncontrolled comparisons must be treated cautiously, they certainly suggest a low renal clearance of EHDP. The experiments were carried out using identical protocols and doses of EDTA and EHDP (mol kg^{-1}). Hypercalcaemia was observed with EHDP and not with EDTA. This is likely to be due to an accumulation of calcium-EHDP complexes in blood resulting from a relatively low renal clearance of EHDP. Additionally, EDTA caused a larger increment in urinary calcium excretion (3.05 ± 0.39 mmol $hour^{-1}$) than did EHDP (1.72 ± 0.48 mmol $hour^{-1}$) though the difference was not significant ($P<0.09$). However if EHDP was excreted by glomerular filtration, as EDTA is known to be (Forland et al 1966), it would be expected that EHDP would cause the greater increase in calcium excretion since it binds almost twice as much calcium per mole as does EDTA. Furthermore, the animals infused with EDTA had considerably lower GFRs than those treated with EHDP. Again, this would result in lower calcium excretion following EDTA administration. Thus it seems likely that EHDP-calcium complexes are only partially filtered by the glomerulus.

Equally, the discrepancies between these two sets of data could be explained by tubular reabsorption of EHDP. This seems less

likely in view of the large size of the EHDP aggregates and their large electrical charge (Wiers, 1971).

In order to further verify that EHDP complexes are partially non-filtrable, it is clearly essential to estimate EHDP in plasma and urine. Unfortunately the only method available (Bisaz, Felix and Fleisch, 1975) requires the use of ^{14}C-EHDP to correct for the large losses inherent in the method. The labelled EHDP was not available and cannot be readily synthesised. It was attempted to determine EHDP in urine as the difference between the inorganic phosphate as measured before and after ashing urine samples in the presence of sodium hydroxide. This method gave excellent recovery of orthophosphate or EHDP added to urine, however the agreement between unadulterated urine with and without ashing was unsatisfactory.

To summarise: EHDP has been shown to form large calcium and magnesium complexes *in vitro* and *in vivo*. These complexes are eliminated by the kidney so producing hypercalciuria and hypermagnesuria. The presence of non-ultrafiltrable calcium and magnesium in plasma and urine indicates that the complexes formed probably pass through the glomerulus. However the extent of the hypercalciuria (compared to that induced by EDTA), and the concomittant hypercalcaemia would support the contention that the clearance of these complexes is less than the GFR.

CHAPTER VII

THE EFFECTS OF CHRONIC ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP) ADMINISTRATION ON THE EXCRETION OF CALCIUM AND MAGNESIUM BY SHEEP AND HORSES

Summary

1. EHDP was administered to horses (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ *per os*) for four days. Blood was collected daily, and 24 hour collections of urine and faeces made. Total and ultrafiltrable calcium and magnesium concentrations were determined in plasma. Endogenous creatinine clearance was also measured. 24 hour faecal calcium and magnesium excretions were measured as total after ashing, and total and ultrafiltrable fractions of faecal water calcium.

2. The two dosage regimes had similar effects on measured variables, allowing the data to be pooled. EHDP did not significantly affect plasma calcium or magnesium plasma fractions, or urinary or faecal magnesium excretion. However the urinary excretion of calcium was increased.

3. EHDP produced significant reductions in 24 hour faecal water. The total and ultrafiltrable faecal water calcium fractions were also reduced, whilst total faecal calcium remained unchanged.

4. Since endogenous creatinine clearance was reduced and ultrafiltrable plasma calcium unchanged, the increment in calcium excretion was thought to be due to decreased renal tubular reabsorption. The effect was too great to be merely due to filtration of calcium-EHDP complexes, possible mechanisms were discussed.

5. Preliminary studies in the sheep, in which measurement of urinary [Ca]/[cr] and [Mg]/[cr] ratios were made, suggested that EHDP (4 mg $kg^{-1} day^{-1}$ by subcutaneous injection) had a similar effect on urinary calcium to that observed in the horse.

Introduction

In Chapter VI evidence was presented which demonstrated that EHDP complexes calcium and magnesium. It was suggested that the hypercalciuria observed following intravenous EHDP was the result of filtration of calcium complexes. In those experiments a large dose of EHDP (40 mg kg^{-1}) was administered over two and a half hours. This dose is much in excess of that at which EHDP has pharmacological actions (Gasser et al, 1972). In man EHDP has beneficial effects in Pagets disease at doses as low as 5 mg kg^{-1} orally (Altman, Johnstone, Khairi, Wellman, Serafini, and Sankey, 1973). Thus, in this chapter the effect of lower doses of the drug was studied.

The dose rates chosen were 4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ *per os*. The 4 mg $kg^{-1}day^{-1}$ dose of EHDP was selected on the basis of its known pharmacological action in cattle (Yarrington et al, 1977b). The extent to which EHDP is absorbed by the gastro-intestinal tract in sheep and horses is not known. Michael et al (1971) estimated that in other species absorption lay in the range 1-15%, higher estimates being obtained from young animals. This range is clearly too large to enable a directly comparable dose to be given *per os*. Based on these figures a dose of 25 mg kg^{-1} was chosen as being unlikely to result in an absorbed dose greater than 4 mg kg^{-1}.

Methods and Results

1. The effects of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection or 25 mg $kg^{-1}day^{-1}$ per os) on plasma, urinary and faecal calcium and magnesium in the horse

Methods

The pH of EHDP solution for subcutaneous injection was adjusted to 7.40 with sodium hydroxide, it was then made approximately isotonic by dilution to give a 4% (w/v) solution. Orally administered EHDP was mixed with sucrose syrup (20 ml, 40% w/v) into which was stirred bran and rolled oats (50 gms, 50/50 w/w).

Orally administered EHDP was fed at 9.00 am each day before the morning hay ration. Parenterally administered drug was divided, 2 mg kg^{-1} was given at 9.00 am and again at 5.00 pm. Initially inflammation frequently followed injection of the drug. In an attempt to aid absorption and so minimise this effect, hyaluronidase (750 i.u., Hyalase, Fisons Ltd) was added and each dose was further divided and given at two sites. Despite these precautions subcutaneous administration of diphosphonate was still occasionally accompanied by an inflammatory reaction. For this reason in subsequent experiments the drug was administered orally.

Seven experiments were carried out in five male ponies. Control experiments consisted of three 24 hour collections of urine and faeces, and twice daily jugular venous blood samples (9.30 am and 4.30 pm). Two weeks later EHDP administration started, after 24 hours a second three day period of collection commenced. EHDP was administered until the end of the experiment. The two ponies which were used twice were allowed 19 weeks between the two experiments.

Ponies were restrained in stalls in which 24 hour faecal and urine collections were made by methods routinely used in this laboratory. Faeces were collected by means of an apron held closely under the pony's tail which directed the faeces into a plastic bin at the rear of the stall. Urine was collected via a large rubber funnel suspended below the pony's abdomen. This delivered urine along a large bore plastic tube into a polythene water carrier situated in a sump below ground level. These devices were attached to the pony by means of a webbing harness and surcingle (Warwick, 1966).

Results

During each 3 day collection period in each animal, since measured variables did not change significantly, means for each variable were calculated so improving precision. Data from the two dosage regimes were then pooled since there were no significant differences between results obtained with either method of administration.

No significant changes in the concentrations of the plasma fractions of calcium or magnesium occurred during the experiment (table 7.1). The 24 hour urinary excretion of calcium increased following EHDP administration ($P<0.01$; figure 7.1) but magnesium excretion did not change significantly. Compared with control, urine volume did not change significantly (control, 2.16 ± 0.18 l 24^{-1} hours; EHDP, 2.44 ± 0.13 l 24 hours^{-1}) whilst GFR decreased ($P<0.05$: control, 247 ± 26 ml min^{-1}; EHDP, 192 ± 19 ml min^{-1}).

Total 24 hour faecal calcium was unaffected by EHDP, but small reductions occurred in both the 24 hour total faecal water calcium ($P<0.01$) and ultrafiltrable faecal water calcium ($P<0.02$; figure

7.2). Total 24 hour faecal water was also reduced ($P<0.05$; figure 7.3) and was reflected in similar changes in faecal weight. The % decrease in faecal water was significantly correlated with both faecal water calcium ($P<0.05$) and with ultrafiltrable faecal water calcium ($P<0.05$; figure 7.4).

Table 7.1

The effect of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ per os) on plasma calcium and magnesium in the horse

mM	Control	EHDP	
$[Ca]_{total}$	2.69 ± 0.08 (7)	2.75 ± 0.06 (7)	ns
$[Ca]_{ultrafiltrate}$	1.51 ± 0.05 (6)	1.58 ± 0.03 (7)	ns
$[Mg]_{total}$	0.62 ± 0.03 (7)	0.65 ± 0.02 (7)	ns
$[Mg]_{ultrafiltrate}$	0.46 ± 0.03 (6)	0.50 ± 0.02 (7)	ns

The results are expressed as mean ± standard error (number of observations). Values were compared with their respective controls. The data for the two dosage regimes were pooled since there were no significant differences between them.

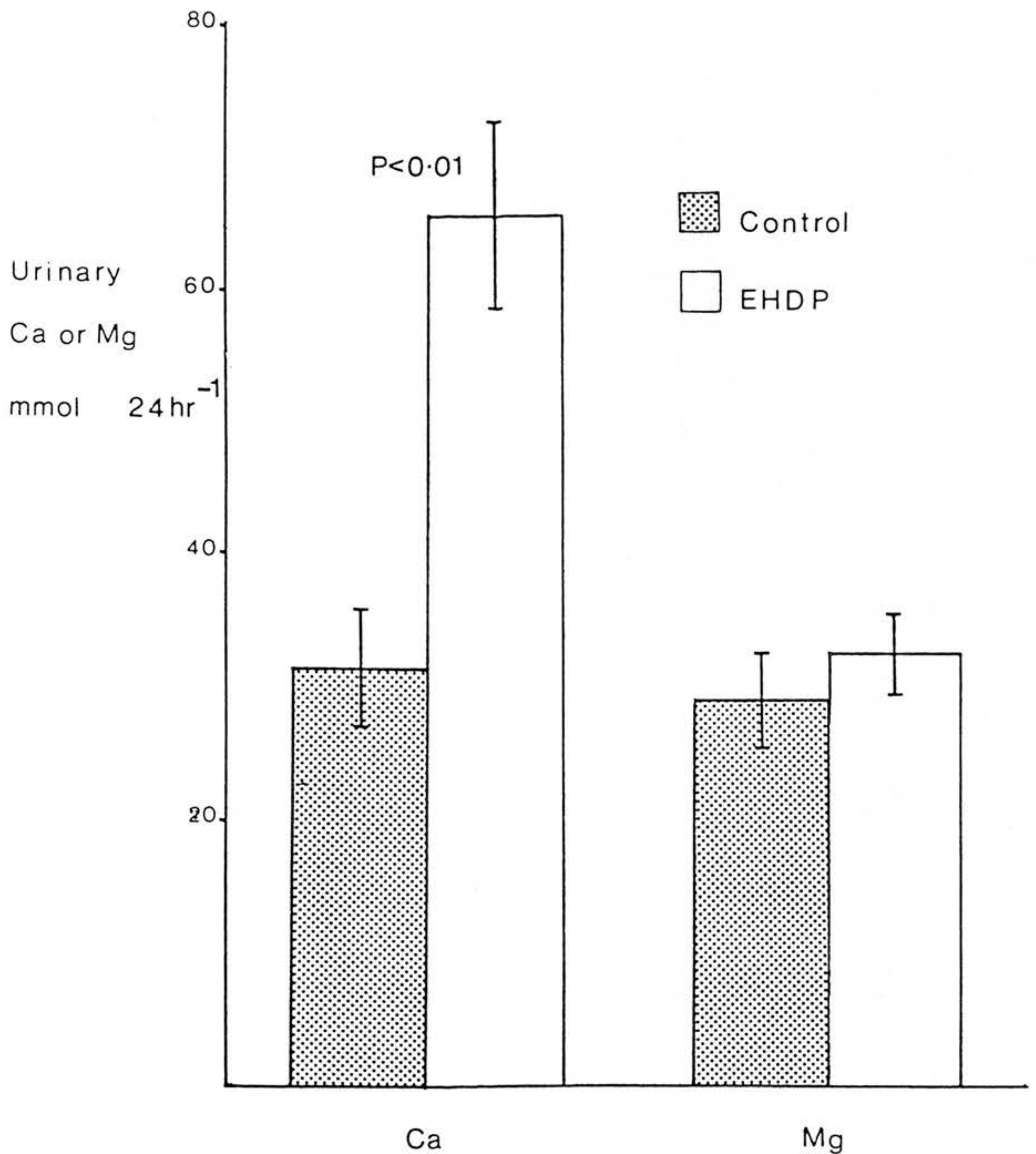


Figure 7.1 The effect of four days administration of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ *per os*) on the 24 hour urinary excretion of calcium and magnesium in the horse. Each bar is a mean and standard error of seven observations.

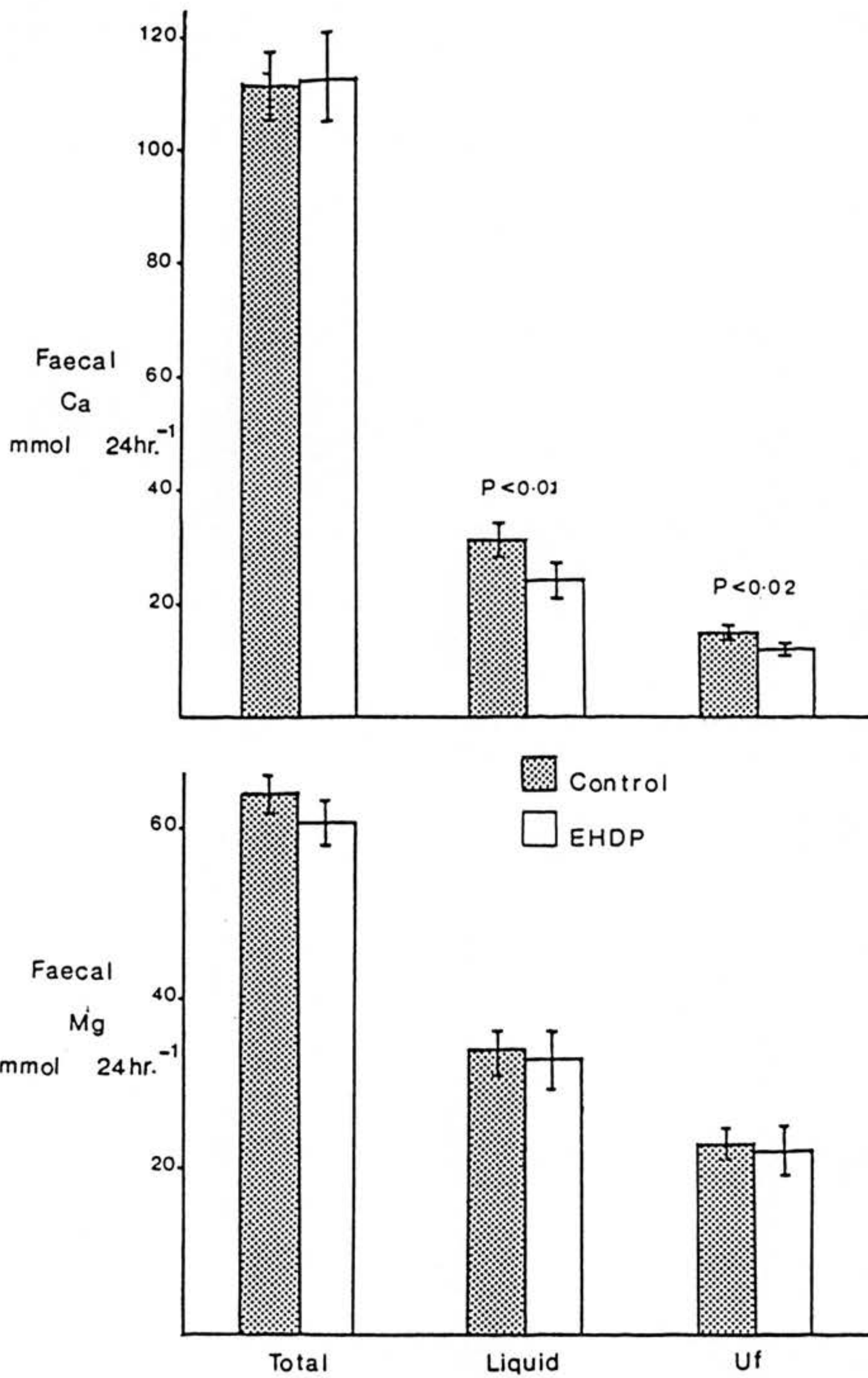


Figure 7.2 The effect of four days administration of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ *per os*) on 24 hour total, liquid phase and ultrafiltrable liquid phase faecal calcium and magnesium in the horse. Each bar is a mean and standard error of seven observations.

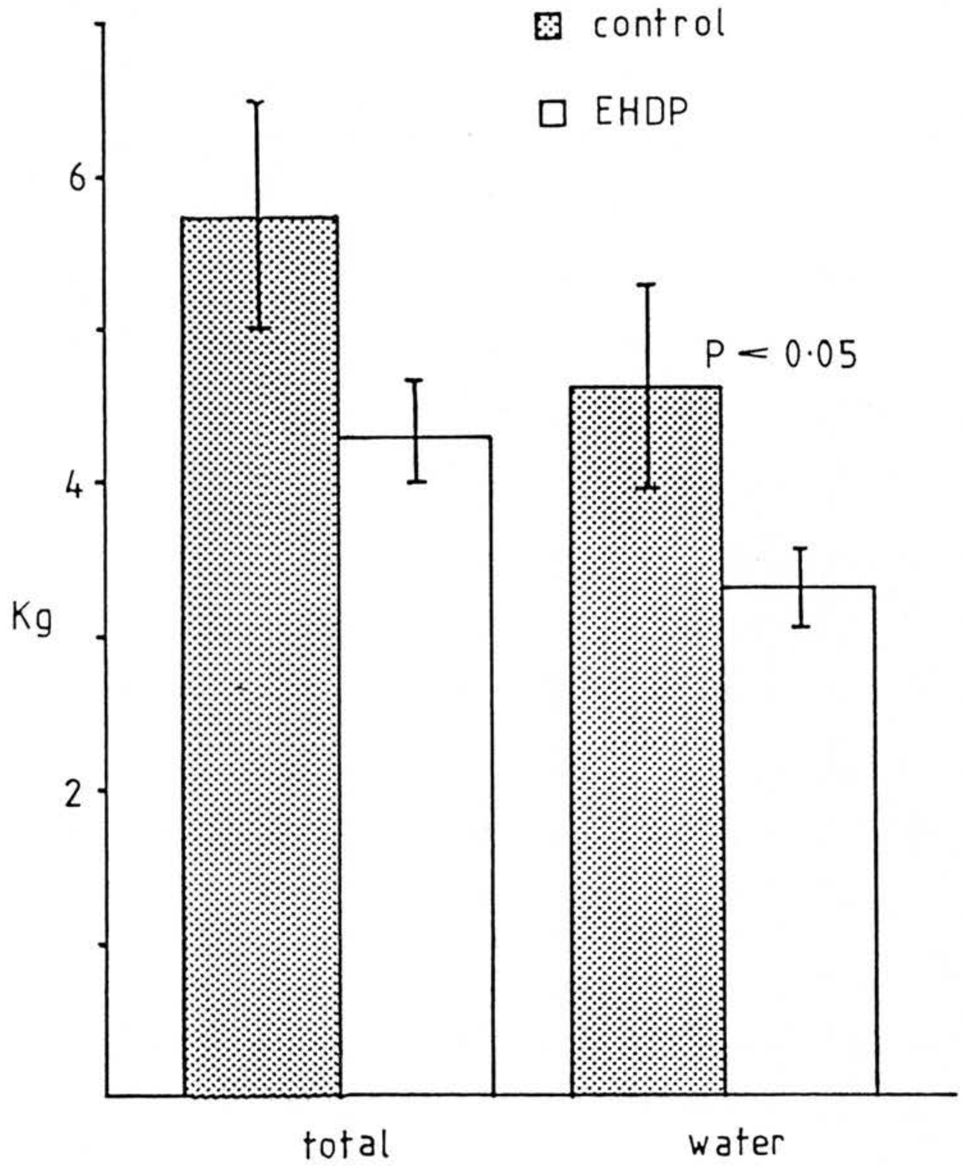


Figure 7.3 The effect of four days administration of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ *per os*) on 24 hour faecal wet weight and faecal water in the horse. Each bar is a mean and standard error of seven observations.

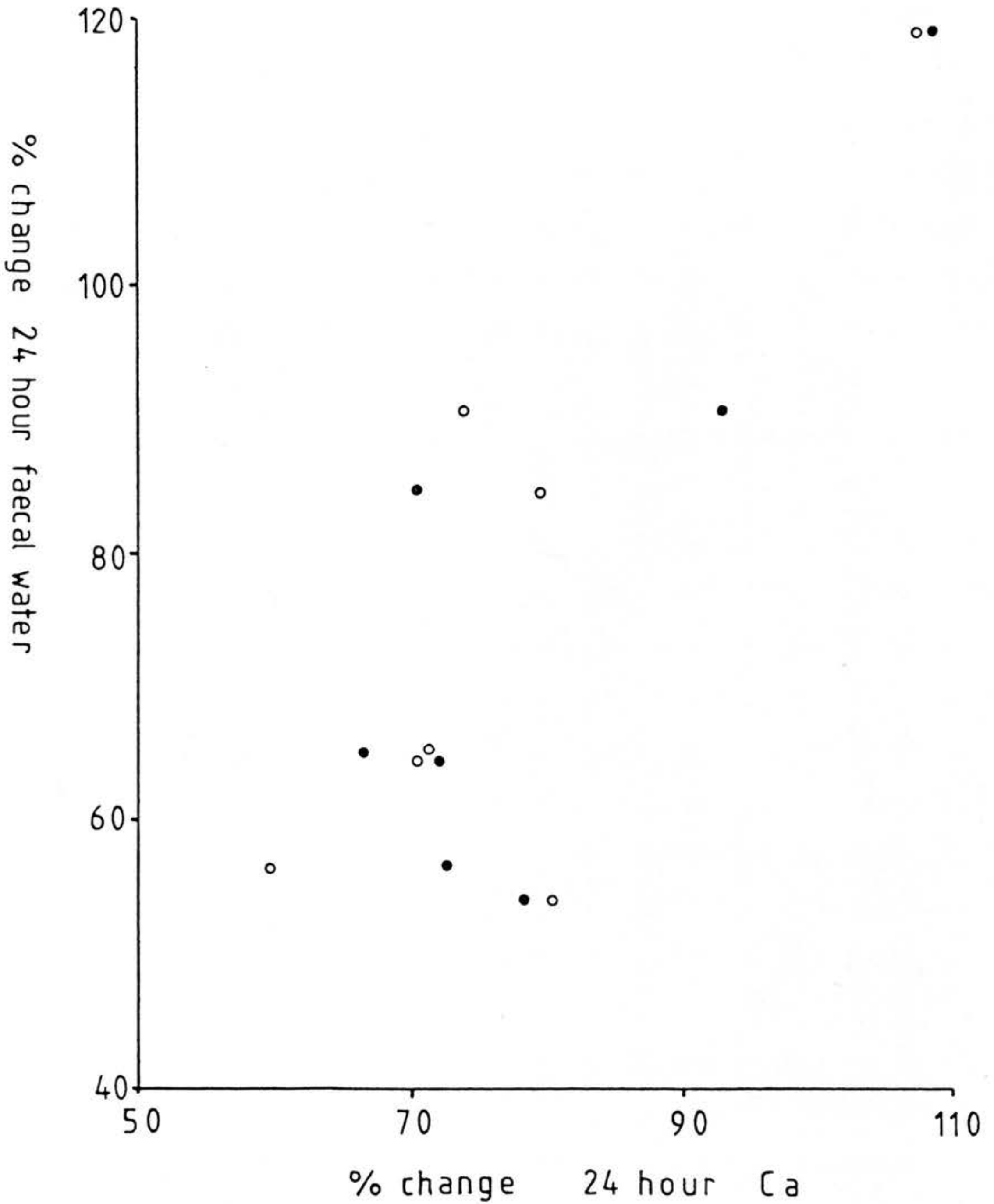


Figure 7.4 The relationship between % change in faecal water and % change in both 24 hour faecal water calcium (open circles), and faecal water ultrafiltrable calcium (closed circles), resulting from EHDP administration (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection, or 25 mg $kg^{-1}day^{-1}$ per os) for four days.

2. The effects of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection) on urinary creatinine (cr) concentration, [Ca]/[cr] and [Mg]/[cr], and plasma calcium and magnesium in sheep

Methods

Four sheep were equipped with indwelling Foley urinary catheters then restrained in crates. Blood samples were drawn into heparinised syringes via jugular puncture at 2.00 pm each day. Urine was collected in plastic urine collection bags via indwelling balloon catheters. Control collections were made for three days, then administration of EHDP (4% w/v pH 7.40; 4 mg $kg^{-1}day^{-1}$ by subcutaneous injection) commenced and continued for a further three days. The experiment was designed to detect sustained rather than transient effects on plasma calcium and magnesium and so EHDP was administered immediately after blood sampling. Thus it was hoped to avoid the acute hypercalcaemia that might result from high EHDP blood concentrations, as described in Chapter VI.

Results

No changes in total plasma calcium or magnesium concentration were observed (table 7.2). Urinary creatinine concentration did not change, but [Ca]/[cr] increased ($P<0.03$); likewise [Mg]/[cr] increased ($P<0.01$). For comparative purposes table 7.3 also includes creatinine concentrations and ratios calculated from the data obtained in the horse.

Table 7.2

The effect of EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection) daily for three days on total plasma calcium and magnesium concentrations in sheep

	Control	EHDP	
[Ca] mM	2.29 ± 0.03 (4)	2.39 ± 0.07 (4)	ns
[Mg] mM	0.729 ± 0.035 (4)	0.700 ± 0.055 (4)	ns

Each value is a mean ± standard error (number of animals).

Table 7.3

Comparison of the effect of EHDP on urinary creatinine concentration, and calcium and magnesium creatinine ratios in the sheep and horse

	creatinine mM		
	Control	EHDP	%change
Sheep	4.94 ± 0.68 (4)	5.51 ± 0.65 (4)	-
Horse	6.08 ± 0.54 (7)	**4.49 ± 0.35 (7)	-26%

	Molar ratio: [Mg]/[cr]		
	Control	EHDP	%change
Sheep	2.64 ± 0.78 (4)	***3.63 ± 0.62 (4)	+38%
Horse	2.26 ± 0.32 (7)	**3.31 ± 0.43 (7)	+46%

	Molar ratio: [Ca]/[cr]		
	Control	EHDP	%change
Sheep	0.26 ± 0.11 (4)	*0.77 ± 0.15 (4)	+196%
Horse	2.34 ± 0.21 (7)	**6.76 ± 1.14 (7)	+189%

Each value is a mean ± standard error (number of observations). Values obtained during EHDP administration were compared with controls: *, $P<0.03$; **, $P<0.02$; ***, $P<0.01$. Where significant changes were observed following EHDP administration, % changes were also calculated.

Discussion

The decreased faecal water calcium observed in the horse might have been due to formation of calcium-EHDP precipitates in the gut. However this seems unlikely since insufficient drug was administered to account for an effect of the observed magnitude. Additionally, the gut concentration of EHDP would be very different after oral or subcutaneous administration, yet the effect on the excretion of faecal water calcium was the same in both cases.

The state of calcium in horse faeces does not seem previously to have been investigated. However, if faeces are normally saturated with respect to calcium, then a reduction in faecal water might result in precipitation of calcium salts and a reduction in total and ultrafiltrable faecal water calcium excretion whilst total was unchanged. This could also account for the observed correlation between faecal water and faecal water calcium.

Alternatively, since the calcium in gut water is probably more readily absorbed than the component associated with the solid phase, changes in calcium absorption might be more readily reflected in this fraction. Thus the change in faecal calcium may be a consequence of increased intestinal calcium absorption, perhaps associated with the concurrent hypercalciuria. Evidence in the rat suggests that EHDP (4 mg $kg^{-1}day^{-1}$ by subcutaneous injection) increases intestinal calcium absorption (Gasser et al, 1972). Balance studies are required to confirm this possibility.

The decrease in GFR observed following EHDP administration to horses has no direct precedent in the literature. Although the ponies showed no clinical signs of abnormality it is possible that changed renal function reflected a mildly toxic action on the

kidney. In the cat oral EHDP at 50 or 500 mg kg^{-1} produced inflammation of both interstitial and tubular elements of the kidney though no effects were seen at lower doses (Jowsey, Holley and Linman, 1970). No such effects have been reported in other species except at very high doses (Nixon, Buehler and Newman, 1972). These reports showed an effect on the tubular elements rather than on the glomerulus.

In the experiments in the sheep complete 24 hour collection of urine was not possible because of intermittent leakage of urine past the catheters. These results must therefore be regarded as of a preliminary nature. However relative changes in excretion may be detected by expressing the urinary concentration of calcium and magnesium as creatinine ratios. Creatinine is a product of muscle metabolism which is produced in proportion to lean body mass. The daily urinary output is thus approximately constant. In man the within individual coefficient of variation of 24 hour creatinine output is 10% (Scott and Hurley, 1968). Though less accurate than complete 24 hour collections, creatinine ratios provide an approximate indication of excretion which is independent of urine volume. Using this method striking similarities in the effect of EHDP on the percentage increase in urinary calcium excretion were seen in the two species.

In this study a relatively low dose of EHDP increased urinary calcium excretion in horses. Since GFR decreased and plasma ultrafiltrable calcium concentration was unchanged the filtered load of calcium was probably reduced. Increased calcium excretion would therefore seem to be due to decreased renal tubular reabsorption of calcium. The change in urinary [Mg]/[cr] seen in the horse merely reflected decreased GFR since 24 hour magnesium

excretion was unchanged. It may be that the change in [Mg]/[cr] observed in sheep also resulted from decreased GFR.

In the experiments described in Chapter VI it seemed that EHDP administration resulted in the formation of calcium-EHDP complexes which were subsequently eliminated by the kidney. The study described in the present chapter used only 10% of the dose used for intravenous infusion in sheep, yet calcium excretion increased without accompanying shifts in plasma calcium fractions.

Calculation shows that, indeed, complexation cannot account for the magnitude of incremental calcium excretion described in the present investigation. Thus, the maximum amount of calcium likely to be excreted in the form of EHDP complexes can be calculated taking the worst case case where 20% of the administered EHDP is absorbed (Michael <u>et al</u>, 1972) and then 50% excreted in the urine (Michael <u>et al</u>, 1972) accompanied by calcium in the ratio 2:1 (Grabenstetter and Cilley, 1971). On this basis, the maximum increase in 24 hour urinary calcium following the administration of 25 mg $kg^{-1}day^{-1}$ EHDP to a 145 kg pony is 3 mmol 24 $hours^{-1}$. In the experiments in the horse the increment in urinary calcium excretion was 34.1 mmol 24 $hours^{-1}$, ten times the predicted value ($P<0.01$). Thus, the increment in urinary calcium cannot be ascribed to excretion of complexes.

Acidosis depresses renal tubular calcium reabsorption and so might account for the observed hypercalciuria. In Chapter VI EHDP was shown to cause a fall in urinary pH, in the present study acid-base status was not evaluated. Alternatively increased calcium excretion may have been related to an increase in intestinal calcium absorption, already suggested as a possible explanation for the observed changes in faecal water calcium.

In conclusion, administration of EHDP to horses depresses renal tubular reabsorption of calcium and thus, despite reduced GFR, produces hypercalciuria. This action was not merely a result of filtration of calcium complexes. The excretion of diffusible faecal calcium was also reduced, in parallel with faecal water. Observations in the sheep suggest a similar phenomenon. EHDP (4 mg $kg^{-1}day^{-1}$) is known to enhance calcium absorption, this phenomenon is one possible explanation for the observed changes in faecal and urinary calcium.

CHAPTER VIII

EFFECT OF AMMONIUM CHLORIDE INDUCED ACIDOSIS AND ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP) ON BONE MINERAL IN THE MOUSE

Summary

1. Mice were given ammonium chloride (0.75% w/v), EHDP (0.03% w/v), or both drugs ad libitum in the drinking water for 290 days. They were killed, then blood pH and femoral dry weight, defatted weight, bone fat and content of calcium, magnesium, sodium and potassium determined.

2. Mice given ammonium chloride developed acidosis. Bone fat and monovalent cations (per gm of defatted bone) were reduced.

3. The administration of EHDP also resulted in reduced bone potassium (both as a total and per gm of defatted bone) compared to the control group. Thus the effects of EHDP and ammonium chloride on bone mineral were similar.

5. It was concluded that EHDP was unlikely to be of value in reducing the effect of acidosis on bone.

Introduction

Goulding and Broom (1979) examined the effect of EHDP on acid base balance. They found that in nephrectomised rats, but not in intact animals, EHDP decreased extracellular and intracellular pH. It was postulated that since EHDP inhibits skeletal mineralisation it may prevent the buffering of acid loads by bone.

Barzel and Jowsey (1969) administered ammonium chloride to rats via the drinking water, this induced a significant loss of organic matrix, bone ash, calcium and phosphorous. Dekanič (1979) found similar reductions in bone ash following calcium chloride administration. Though it was not certain how reproducible these observations might be (Newell *et al,* 1975), acidosis induced changes in bone might serve as a long term model for the study of bone calcium, thus facilitating investigations of the ability of EHDP to prevent demineralisation.

It was therefore proposed to examine the effects of EHDP on bone mineral in normal and acidotic mice.

Methods

Random bred CF1 mice, five months old, were weighed and randomly allocated to provide four groups of equal size. They were fed on a standard diet (BP Nutrition No. 1 modified expanded maintenance diet) containing (w/w) 0.83% calcium, 0.51% phosphorous, and 0.15% magnesium. Drinking water containing ammonium chloride (0.75% w/v), EHDP (0.03% w/v) or both drugs was provided *ad libitum* to each of three groups. The fourth group which served as the control received distilled water. The drinking water was provided in standard plastic water bottles, they were closely checked for leaks enabling measurement of the water consumption to be made over the last 70 days of the experiment.

After 290 days the mice were anaesthetised with ether then exanguinated by incising the axilla through the axillary artery and vein. The mixed arterio-venous blood which rapidly filled the incision was drawn into a heparinised syringe. Exposure to air and subsequent loss of CO_2 was minimised by making a deep incision, so forming a well into which the blood could flow whilst exposing a small surface area. This procedure usually yielded 0.4-1 ml of blood. Blood pH was measured as described in the Chapter II, with the exception that the micro-sampler attachment of the Corning blood gas analyser was used. This reduced the volume of blood required to 0.2 ml. Bones were weighed, defatted, weighed again, then ashed and their calcium, magnesium, sodium and potassium content measured.

Results

Ten deaths occurred during the experiment, all 6 deaths in the control group occurred on one occasion and were the result of an air-lock in the water bottle. The cause of the 3 deaths in the NH_4Cl group and the 1 death in the EHDP group was thought to be the same, though this was not certain.

Over the last 70 days of the experiment the water consumption in the four groups was 2.8 $\pm$ 0.1 ml mouse^{-1}day^{-1} (mean $\pm$ standard error). The mean weight of the animals was 26.1 gm and so the calculated daily doses were 15 mmol kg^{-1} ammonium chloride and 30 mg kg^{-1} EHDP.

At both the beginning and end of the experiment, the weights of the four groups of mice did not differ significantly (table 8.1). The weight of the control group increased during the course of the experiment ($P<0.05$) but none of the other groups showed significant changes in body weight.

As expected, the administration of ammonium chloride reduced blood pH compared to either the control ($P<0.01$) or EHDP treated groups ($P<0.03$) (table 8.1). The simultaneous administration of EHDP and ammonium chloride was also associated with acidosis when compared with either the control ($P<0.002$) or EHDP treated mice ($P<0.003$). The administration of EHDP had no significant effect on blood pH in normal mice or mice with ammonium chloride induced acidosis.

Analysis of variance revealed significant differences in total bone fat and potassium. It also showed differences in bone sodium and potassium per gm defatted bone (table 8.3 and 8.4). Treatment with EHDP reduced total femoral potassium content ($P<0.001$) (table 8.3)

compared to the control group. The ammonium chloride and ammonium chloride plus EHDP mice showed no significant changes from control in either femoral weight or the total content of any cation. Femur fat content was reduced by ammonium chloride compared to either the control ($P<0.001$) or EHDP ($P<0.001$) treated groups of animals.

When femoral cation content per gram of defatted bone was examined (table 8.4) other changes were revealed. Bone potassium was still significantly reduced by EHDP ($P<0.001$). Additionally, ammonium chloride depressed femoral potassium per gm when administered either with ($P<0.001$) or without ($P<0.01$) EHDP. Ammonium chloride also depressed bone sodium per gm both in the presence ($P<0.001$) or absence ($P<0.05$) of EHDP. Total measured cations per gram of bone were not significantly altered by any of the treatments compared to the control.

Table 8.1

The body weights of mice before and after treatment with EHDP (0.03% w/v) and ammonium chloride (0.75% w/v), administered ad libitum in the drinking water for 290 days

	Control	NH_4Cl	EHDP	NH_4Cl and EHDP
Initial body wt. gm	23.4 ± 1.0 (19)	23.5 ± 0.8 (19)	21.5 ± 2.1 (19)	24.4 ± 1.1 (19)
Final body wt. gm	27.7 ± 1.7 (13)	25.1 ± 1.5 (16)	*24.0 ± 0.6 (18)	27.8 ± 1.5 (19)
Significance of difference	$P<0.05$	ns	ns	ns

Figures are means ± standard errors (number of observations). The significance of differences between initial and final weights for each treatment group is shown in the table. The final weights for each treatment were also compared with the control group: * $P<0.03$.

Table 8.2

The changes in blood pH in groups of mice treated for 290 days with EHDP (0.03% w/v) and ammonium chloride (0.75% w/v) administered ad libitum in the drinking water

	Blood pH	Significance compared with: Control	NH_4Cl	EHDP
Control	7.34 ± 0.04 (8)	-	-	-
NH_4Cl	7.25 ± 0.06 (14)	P<0.01	-	P<0.03
EHDP	7.31 ± 0.02 (17)	ns	P<0.03	-
NH_4Cl and EHDP	7.23 ± 0.02 (17)	P<0.002	ns	P<0.003

Figures are means ± standard errors (number of observations).

Table 8.3

Bone dry weight, fat and cation content of the left femurs of groups of mice treated for 290 days with EHDP (0.03% w/v) and ammonium chloride (0.75% w/v), administered ad libitum in the drinking water

	Control (13 animals)	NH_4Cl (16 animals)	EHDP (18 animals)	NH_4Cl and EHDP (19 animals)	Analysis of variance
Bone dry wt. (mg)	41.8 $\pm$ 1.7	40.9 $\pm$ 1.6	38.4 $\pm$ 0.9	42.4 $\pm$ 1.4	ns
Defatted wt. (mg)	39.7 $\pm$ 1.6	40.0 $\pm$ 1.5	36.5 $\pm$ 0.9	41.6 $\pm$ 1.4	ns
Bone fat wt. (mg)	2.14 $\pm$ 0.12	a,*0.90 $\pm$ 0.13	1.87 $\pm$ 0.10	a,*0.88 $\pm$ 0.12	$P<0.001$
Calcium (micro-mol)	238 $\pm$ 9	237 $\pm$ 8	227 $\pm$ 6	249 $\pm$ 13	ns
Magnesium (micro-mol)	6.38 $\pm$ 0.29	6.12 $\pm$ 0.21	5.76 $\pm$ 0.17	6.53 $\pm$ 0.31	ns
Sodium (micro-mol)	9.89 $\pm$ 0.38	9.42 $\pm$ 0.39	8.84 $\pm$ 0.25	9.39 $\pm$ 0.33	ns
Potassium (micro-mol)	1.97 $\pm$ 0.13	1.62 $\pm$ 0.15	*1.27 $\pm$ 0.11	1.64 $\pm$ 0.13	$P<0.001$

Figures are means $\pm$ standard errors. Analysis of variance was applied to each group of measured variables. When significant differences were thus observed between treatments, the un-paired t-test was applied to to all combinations of treatments. Differences were observed compared to control: *, $P<0.001$; and compared to EHDP treated groups: a, $P<0.001$.

Table 8.4

Cation content (per gram of defatted bone) of the left femurs of groups of mice treated for 290 days with EHDP (0.03% w/v) and ammonium chloride (0.75% w/v), administered ad libitum in the drinking water

	Control (13 animals)	NH_4Cl (16 animals)	EHDP (18 animals)	NH_4Cl and EHDP (19 animals)	Analysis of variance
Calcium (mmol gm^{-1})	6.01 ± 0.10	5.94 ± 0.08	6.23 ± 0.09	5.97 ± 0.10	ns
Magnesium (micro-mol gm^{-1})	161 ± 3	153 ± 2	158 ± 2	157 ± 4	ns
Sodium (micro-mol gm-1)	250 ± 3	*236 ± 6	242 ± 3	a,***226 ± 3	P<0.001
Potassium (micro-mol gm-1)	49.3 ± 1.4	**40.1 ± 2.6	***34.7 ± 2.9	***38.8 ± 2.1	P<0.001
Total measured cations (mmol gm^{-1} defatted wt)	6.47 ± 0.10	6.37 ± 0.09	6.66 ± 0.09	6.39 ± 0.10	ns

Figures are means ± standard errors. Analysis of variance was applied to each group of measured variables. When significant differences were thus observed between treatments, the un-paired t-test was applied to to all combinations of treatments. Differences were observed compared to controls: *, P<0.05; **, P<0.005, ***, P<0.001; and compared to the EHDP treated group: a, P<0.001.

Discussion

In these studies acidosis did not significantly alter total femoral content of any of the measured cations whilst EHDP lowered total femoral potassium content. However the value of total cation content as a means for comparison was questionable because of the relatively large variation in body weights between the groups of animals at the end of the experiment. This was reflected by similar variability of dry and defatted femur weights. Although not statistically significant, such variability may bias the data. In order to overcome this problem the results were also expressed as cations per gram of defatted bone. The coefficient of variation for many of the measured variables was greatly reduced by examining the data in this way.

In the present work, despite a reduction in blood pH, ammonium chloride had no effect on total femoral calcium content. In contrast, studies in rats demonstrated significant reductions (Barzel and Jowsey, 1969). Despite the higher ammonium chloride concentration used by Barzel et al (1969), as mice drink considerably more water (240 ml kg^{-1}) than rats (80 ml kg^{-1}) (Bruce, 1950) the ingested dose was probably greater in the present study.

It is not clear why bone calcium content was unaffected, it may be that the mouse has a greater ability than the rat to compensate for an acid load by mechanisms such as the production of urinary ammonium (Janicki and Argyris, 1969). However the relatively large fall in blood pH is evidence against this possibility and suggests rather that mice femora are, perhaps, relatively resistant to dissolution by acidosis.

As in the present study, Barzel et al (1969) did not find that ammonium chloride altered total bone sodium or potassium. However, they did not examine sodium or potassium per gm of bone. Burnell (1971) examined changes in bone mineral in dogs by relating the content of various ions to calcium. Thus he found significant reductions in bone sodium and carbonate to calcium ratios in dogs after only 10 days of ammonium chloride treatment. Though potassium was not examined, depression of sodium content was similar to the present study.

The cause of the large reduction in total femoral fat content following ammonium chloride administration is uncertain. Barzel et al (1969) found reductions in the organic phase of bone, but did not examine changes in bone fat content.

The studies clearly demonstrated that EHDP depresses femoral potassium content. It is not surprising that EHDP (30 mg $kg^{-1}day^{-1}$) altered the composition of bone, in view of the increased amount of non-mineralised osteoid tissue seen in man following prolonged administration at doses greater than 20 mg $kg^{-1}day^{-1}$ (Jowsey et al, 1971; Russell and Fleisch, 1975). Short term experiments in animals also demonstrated increased non-mineralised osteoid tissue (King, Francis and Michael, 1971; Rosenblum, 1974). Determination of whole bone mineral content is clearly very difficult in experimental studies in man. The only comparable study in animals is that of Rosenblum (1974) who did not examine bone monovalent cations and failed to find any change in bone calcium and phosphorous content following 28 days of EHDP treatment in rabbits. There is thus little published evidence which assists discussion of the observed effects.

It is likely that acidosis would modify the pharmacology of

EHDP. The pKs of three of the equivalence points of EHDP are 11.5, 7.3 and 3.0 (Grabenstetter, Quimby and Flautt, 1967). Since the pK of one of the hydroxyl groups is so close to 7.4, blood pH changes of the order seen here (0.1 pH units) would be expected significantly to alter the ionization and hence the absorption and excretion of EHDP. Since EHDP complexes are more dissociated at low pH and since EHDP probably acts, at least in part, by physically binding onto bone, lowered extracellular pH might also be expected to reduce the efficacy of EHDP.

However the changes in bone potassium following EHDP were no different during simultaneous administration of ammonium chloride, despite demonstrable acidosis. In fact EHDP and ammonium chloride had very similar effects. The greater depression of femoral sodium, (per gm of defatted bone) in the ammonium chloride and EHDP treated group, as compared with the animals treated with EHDP or ammonium chloride alone perhaps indicates additive effects. Certainly, EHDP does not moderate the detected bone changes induced by acidosis. If bone acid-base buffering is chiefly achieved by loss of carbonate and bicarbonate, together with their associated monovalent cations (Burnell, 1971), then these results are at variance with the suggestion that EHDP reduces the ability of bone to buffer an acid load (Goulding and Broom, 1979). Other diphosphonates, such as dichloromethylene diphosphonate, which act more specifically on resorption, may prove of greater value in the prevention of acidosis induced de-mineralisation.

CHAPTER IX

GENERAL DISCUSSION

In the preparation of this thesis a considerable effort was devoted to the development and validation of methods of measuring calcium and magnesium fractions in plasma. The importance of these fractions has been amply discussed and their lability demonstrated by the results described in Chapter III which clearly indicates the importance of validating such methods.

The method of preparing plasma ultrafiltrates seemed to be susceptible to temperature and Donnan shifts. Small shifts in pCO_2 and pH occurred during the procedure, though with care these changes could be reduced so as to have a minimal effect on ultrafiltrable calcium and magnesium concentrations. Similarly, ionized calcium was found to be affected by heparin concentration. Careful control ensured that heparin concentration had a constant but very small effect on ionized calcium.

Unfortunately it was not possible to develop a suitable method of measuring plasma ionized magnesium. The study of Heaton (1967) has hitherto been widely accepted as providing acceptable reference values for ionized magnesium. The studies described in this thesis clearly indicate that this is not the case. In so far as Heaton (1967) investigated his method, the present studies agree with his findings. However, more detailed investigation demonstrated that the method does not distinguish adequately between ionized and chelated magnesium.

None of the published methods seemed to offer alternatives

suitable for routine determinations. For example, spectrophotometric methods are relatively cumbersome and, more seriously, involve dilution and buffering of plasma ultrafiltrates. This is likely to lead to considerable errors. Other ion exchange methods (Frizel et al, 1967), like that of Heaton (1967), may also register a proportion of complexed cations as free ions.

In this thesis, intravenous infusions of EDTA in sheep had the primary effects expected of a complexing agent: decreased ionized plasma calcium, and increased urinary calcium excretion. The increase in complexed plasma calcium concentration was similar in magnitude to the EDTA concentration. Similarly the increments in urinary calcium and EDTA were equimolar.

EHDP infusion had similar effects on calcium kinetics: increased urinary calcium excretion and decreased ionized plasma calcium. These observations suggested complex formation was occurring *in vivo,* as demonstrated *in vitro.* However two differences were noted between EDTA and EHDP. Firstly, whereas with EDTA the increments in plasma and urinary calcium were due to changes in the ultrafiltrable fraction, with EHDP increases in chelated calcium in plasma and urine were due to increases in the non-ultrafiltrable fraction. Thus it seemed that EHDP, unlike EDTA, was forming complexes of relatively large molecular weight. Secondly, EHDP quite clearly complexed significant amounts of magnesium *in vitro* and *in vivo*, as evidenced by changes in non-ultrafiltrable plasma and urinary magnesium. Prior evidence for magnesium binding could not be found in the literature.

Since the decrease in ultrafiltrable calcium and magnesium was the same in plasma and saline solutions containing EHDP, the non-ultrafiltrable nature of calcium and magnesium in plasma could

not be related to protein binding of EHDP-cation complexes. It probably, therefore, resulted from the formation of large aggregates.

Methods of determining ultrafiltrable calcium and magnesium use relatively crude filters. However attempts to produce filters which are comparable to the glomerular filter are fraught with difficulties. Normally calcium and magnesium in plasma are bound primarily by plasma proteins and ligands of low molecular weights (such as citrate and phosphate). Thus, providing the membrane retains protein yet filters small molecules freely, the measured ultrafiltrable calcium and magnesium will reflect the true glomerular calcium and magnesium concentrations. However once substances of intermediate molecular weight are introduced errors will occur. The behaviour of the glomerulus with respect to a variety of substances of varying molecular weight has been investigated. Giebisch _et al_ (1954) summarised data from a variety of sources; the relationship between molecular weight and filtered fraction is shown in figure 9.1.

It seems that the renal clearance of partially filtrable substances of intermediate molecular weight cannot be adequately characterised using artificial membranes. Thus the measured ultrafiltrable calcium and magnesium in the presence of EHDP does not pertain to the situation _in vivo_. However, _in vitro_ studies (Wiers, 1971) suggest that the size of EHDP complexes is such that they are likely to be only partially filtrable by the glomerular filter (as defined by the review of Giebisch _et al_, 1954). The low renal clearance of ^{99m}-technetium-EHDP has been demonstrated (Hughes _et al_, 1977). The results of the studies reported in Chapter VI also suggest low clearance, especially when compared

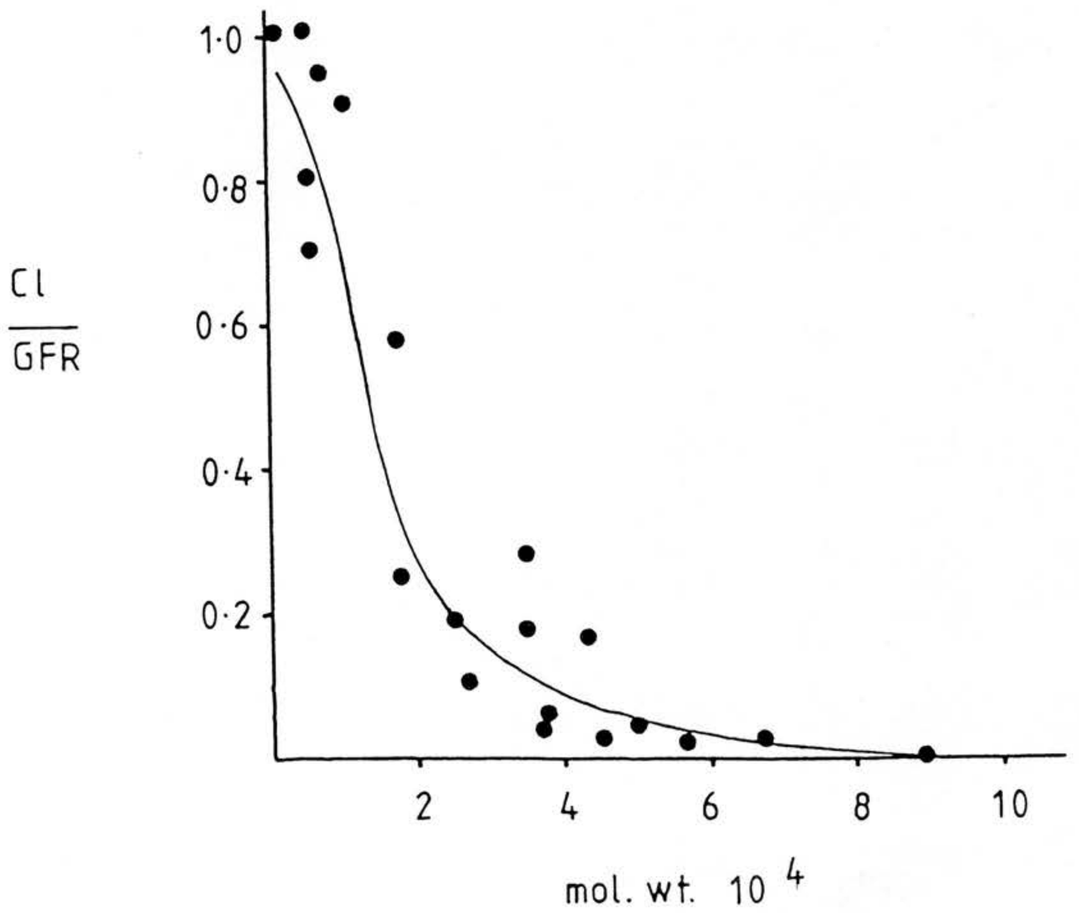


Figure 9.1 The ratio of renal clearance/GFR as a function of molecular weight for a variety of substances (dextrans, haemoglobin, myoglobin, gelatin, polyethylene glycols, grass polysaccharides, ovalbumin, ovomucoid, B-lactoglobulin and lysozyme. Adapted from Giebisch, Lauson and Pitts, 1954).

with the results of EDTA infusion.

Prior to the studies reported here, no evidence could be found relating to the ionization of calcium borogluconate. In this work the non-ionized nature of the solution has been clearly demonstrated. The infusions of calcium borogluconate were less readily comparable with those of EDTA or EHDP since the calcium salt was used. As a result, different changes in blood and urine levels of calcium and magnesium were seen. Thus, although urinary calcium excretion increased, any effects due to ligand binding were obscured by the concurrent hypercalcaemia. For the same reason, calcium borogluconate infusion, unlike EDTA and EHDP, induced pronounced increases in all three fractions of plasma calcium. Much of the increment in plasma calcium was due to an increase in ultrafiltrable, non-ionized calcium. Thus the complexes were relatively small compared to those formed by EHDP. Administration of sodium borogluconate depressed total plasma calcium, EHDP infusion had the opposite effect. This difference was probably due to the lower rate of excretion of EHDP as a result of its poor clearance.

In the treatment of hypocalcaemia, the disadvantages of anions that complex calcium are clear. Much of the rise in blood calcium will be ineffectual and ligands which are readily filtered will enhance the renal excretion of calcium. The use of anions which, unlike borogluconate, are not filtered appreciably by the glomerulus would help to retain calcium in the circulation. Such substances could prove valuable in the treatment of clinical hypocalcaemia providing the equilibrium constants were such that calcium was readily released at low plasma ionized calcium concentration and physiological pH. Blood and Henderson (1974)

mention the use of calcium carboxymethyl-dextran. If calcium is significantly bound by this anion, then it might prove of value.

The ability of endogenous ligands to buffer changes in ionized calcium following addition of calcium chloride or EDTA to blood was clearly demonstrated in the evaluation of the Nova-2 calcium electrode described in Chapter III: only 50% of added or removed calcium came from the ionized fraction. In a similar way exogenous ligands, such as borogluconate, might regulate ionized calcium by complexing calcium and releasing it in inverse proportion to the plasma ionized calcium concentration. Hydrogen ion buffers are most effective when the pH of the solution is close to the pK of the buffer. Similarly, significant benefits with calcium ion buffers will only be gained when the pCa of the solution is close to the pK_{Ca} of the buffer. It would also be necessary for the binding constant to be appropriate; a ligand such as EDTA would clearly be useless *in vivo*. During therapeutic infusions of calcium salts, anions with such properties would prevent excessive rises in plasma ionized calcium which might otherwise occur with a subsequent risk of acute myocardial toxicity. Indeed, the studies reported in Chapter V indicated that when small amounts of calcium borogluconate were added to plasma, considerably more of the calcium was ionized than when large amounts were added. This may represent a significant advantage of calcium borogluconate. The metabolism of such anions would also affect their value.

These experiments suggest that the action of borogluconate on calcium kinetics deserves further attention. Although the advantages and clinical efficacy of calcium borogluconate are not in dispute, comparative evidence obtained using a variety of with calcium salts might prove useful. Since 25-30% of treated cases of

hypocalcaemia relapse (Blood, Henderson and Radostits, 1979), salts giving improved retention of calcium may offer a significant advantage in the treatment of hypocalcaemia.

EHDP is rather different from EDTA and borogluconate in that it clearly has other actions in addition to its ability to alter the kinetics of calcium and/or magnesium merely by complexation. Thus, the evidence presented in Chapter VII demonstrated a hypercalciuric action which, on a stoichiometric basis, could not be accounted for by complexation. The mode of action of this effect was uncertain though it may have resulted from enhanced calcium absorption from the gut.

The major interest in diphosphonates stems from their ability to decrease the rate of bone turnover. This property could be valuable in the treatment of many conditions in man and animals. It is particularly interesting that in the treatment of Paget's disease (a proliferative bone disease in man associated with increased bone turnover), remission from disease persists long after the cessation of treatment. However, although in certain circumstances EHDP reduces demineralisation (Michael et al, 1971; Lindenhayn et al, 1982), the production of beneficial rather than deleterious effects clinically, requires careful control of dosage (Rashid, Khairi and Johnston, 1977).

The future probably lies with other diphosphonates which have a lesser tendency to cause loss of bone substance. Dichloro-methylene diphosphonate is one such drug (Gasser et al, 1972), but the most promising agent at present seems to be 3-amino-1-hydroxy-propylidene-1,1-diphosphonate (ADP) (Lemkes, Reitsma, Frijilink, Verlinden-Ooms and Bijvoet, 1977). This drug reduces both mineralisation and resorption but seems to have a lesser tendency

to cause de-mineralisation than EHDP. Preliminary studies suggest that it may prove useful in man (Frijlink, Bijvoet, Velde and Heynen, 1979).

In this thesis the actions of some drugs which complex calcium and magnesium have been studied. Their ability to alter partition of calcium and magnesium between plasma compartments and, as a result of reduced bio-availability, enhance excretion has been clearly demonstrated. In addition some more novel actions have been studied, such as alterations in bone turnover. The latter actions are less clearly the result of complexation *per se*, and may result from more specific pharmacological actions of the drugs.

Appendix

A comparison between the renal clearance of endogenous creatinine and inulin in the sheep

Summary

1. Intravenous infusions of inulin were made in four sheep. Urine was collected for periods of one hour and blood at the beginning and end of each period. Simultaneous renal clearances of inulin (Cl_{in}) and endogenous creatinine (Cl_{ecr}) were determined during periods of stable plasma inulin concentration.

2. Cl_{ecr} was found to be a reasonable method of measuring GFR in the normal range. However the calculated regression of Cl_{ecr} on Cl_{in} differed slightly from the expected line: at high clearances Cl_{ecr} was slightly greater than Cl_{in}, at low clearances the reverse was true. When glomerular filtration rate (GFR) was in the range 14-48 ml min^{-1}, encompassing the majority of observations made, the two clearances did not differ significantly.

3. It is suggested that the small differences between creatinine and inulin clearance were due to lack of specificity of the method used for creatinine determination. The use of Cl_{ecr} introduces errors at high or low GFR, but these are insignificant compared to the normal variability of clearance methods.

Introduction

Inulin has been found to be a more suitable marker than any other substance studied for the measurement of GFR. Inulin is neither secreted nor reabsorbed by the renal tubule (Levinsky and Levy, 1973). Creatinine is also a useful indicator of GFR in the dog cat, seal and rabbit, however it is secreted by the proximal renal tubule in man, primates, dogfish, teleosts and chickens (Smith, 1951) and so cannot be used in these species. Two perfunctory comparisons of creatinine and inulin clearance in sheep have been published (Shannon,1937; Schmidt-Nielsen, Osaki, Murdaugh and O'Dell, 1958).

Creatinine clearance is measured either during creatinine infusion (exogenous creatinine clearance; Cl_{cr}) or by utilising endogenous creatinine (Cl_{ecr}). On a herbivorous diet endogenous creatinine is derived almost entirely from the breakdown of muscle phosphocreatines and creatine, the amount produced is therefore closely related to lean body mass. For long term experiments endogenous Cl_{ecr} is the most practicable method of assessing GFR.

The chromogens in plasma which are measured as creatinine comprise the "true" and "pseudo" creatinine fractions. The latter component includes many different substances. Lauson (1951) showed that non creatinine chromogens in plasma accounted for 25-75% of the Jaffé positive substances[1] in plasma. The presence of "pseudo" creatinines forms the major drawback to the use of endogenous creatinine clearance since it is important that only substances

[1]The Jaffé reaction is the alkaline picrate reaction used for the determination of creatinine, it measures both "true" and "pseudo" creatinine, unless used in conjunction with an adsorption technique.

which are filtered without secretion or reabsorption are measured. This problem can be overcome either by infusing creatinine so as to raise the plasma creatinine sufficiently that "pseudo" creatinine concentration becomes negligible, or by using a method of creatinine estimation which eliminates the "pseudo" creatinines.

Narayanan and Appleton (1972) found that methods of measuring "true" creatinine, including the one used in this study, were not entirely specific for creatinine. However providing the method used measures substances which are excreted without reabsorption or secretion, it will be suitable for the determination of GFR. Thus, despite evidence that a method is not specific, reliance may be placed on an examination of the relationship between either Cl_{cr} or Cl_{ecr} and Cl_{in} in evaluation of its suitability.

Knudsen (1959) studied creatinine clearance in the horse and found that both Cl_{ecr} and Cl_{cr} were closely related to inulin clearance. However, the values of Cl_{ecr} were 20% higher than the simultaneously measured Cl_{in}.

Shannon (1937) and Schmidt-Nielsen et al (1958) are the only investigators to have made simultaneous determinations of creatinine clearance and Cl_{in}. Shannon (1937) measured Cl_{cr} in one sheep, Schmidt-Nielsen et al (1958) measured Cl_{ecr} in two sheep during a large number of clearance periods. No significant differences were observed between creatinine and inulin clearances in either of these investigations.

Since so little work had been done to verify the validity of Cl_{ecr} as an estimate of GFR in the sheep it was proposed to compare the renal clearances of inulin and endogenous creatinine. Subsequently it was hoped to make extensive use of Cl_{ecr} to estimate GFR.

Methods

Sheep (21-43 kg weight) were catheterised for urine and blood collection, and for intravenous infusions as described in Chapter II. Before the start of an infusion samples of blood and urine were taken as inulin blanks. Inulin was then infused at a rate of 1 mg $kg^{-1}min^{-1}$ following a priming dose of 17 mg kg^{-1}. Infusion was started at least one hour before clearances were measured, thus clearances were only determined during periods of stable plasma inulin concentration. Four sheep were used in this experiment, two sheep were used on two separate occasion thus providing a total of six infusions. Clearances were calculated from hourly urine collections, and the mean of the concentrations of creatinine or inulin in blood samples taken at the beginning and end of each clearance period.

Results

The gradient of the regression of Cl_{ecr} on Cl_{in} differed from 1.00 ($P<0.005$; figure 1a). The intercept differed from 0.00 ($P<0.02$). The ratio Cl_{ecr}/Cl_{in} was correlated with the plasma concentrations of both creatinine ($r=0.79$, $P<0.001$) and inulin ($r=0.77$, $P<0.001$). No significant correlations were observed between urine volume and Cl_{ecr} ($r=0.32$), or urine volume and Cl_{in} ($r=0.28$). Over the range of Cl_{in} 14-48 ml min^{-1}, Cl_{ecr} and Cl_{in} were not significantly different. The within infusion coefficient of variation for all inulin clearance periods was 14.5 $\pm$ 3.6% (mean $\pm$ SEM).

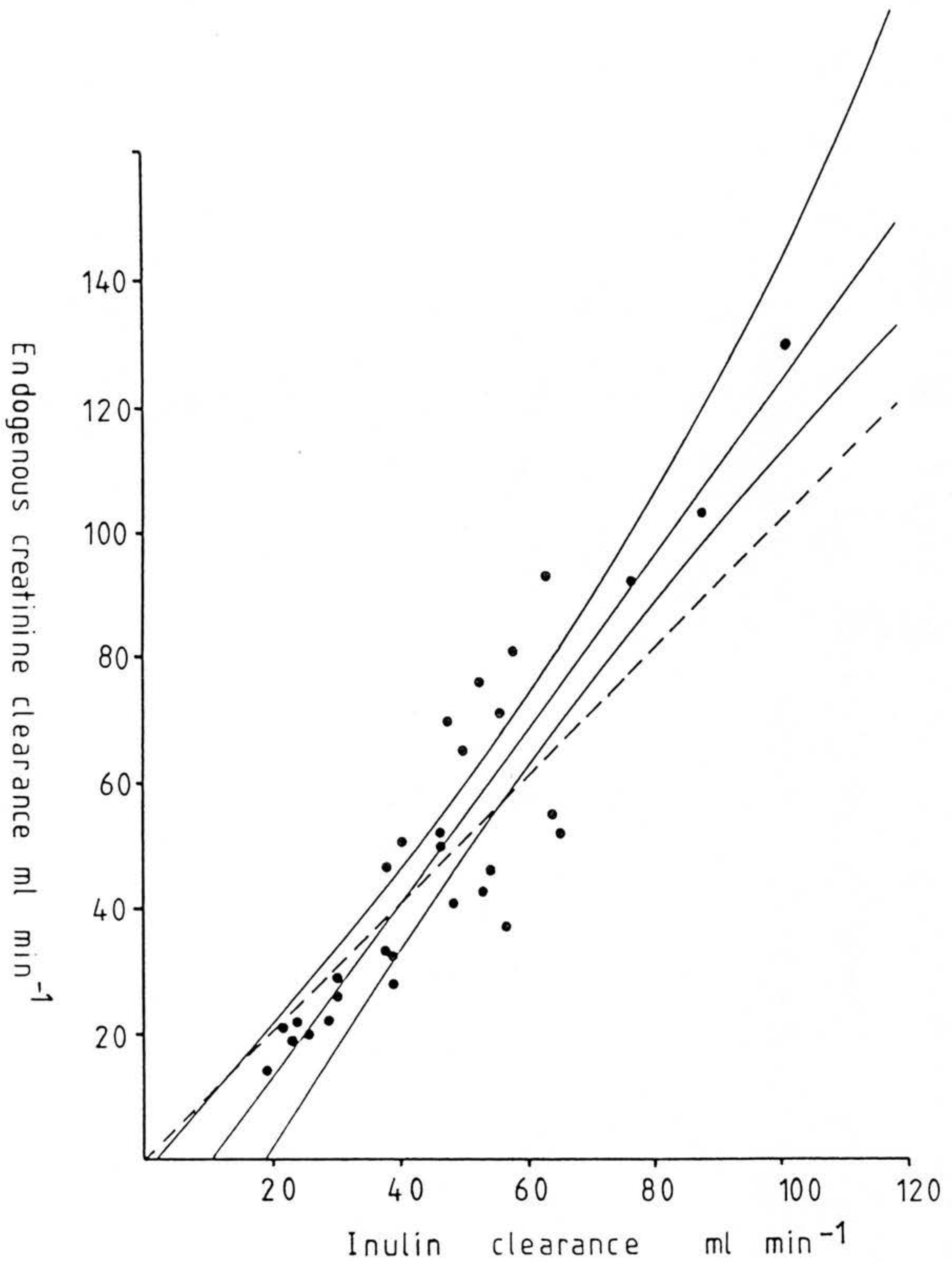


Figure 1a Comparison of simultaneous measurements of the renal clearances of inulin and endogenous creatinine in four sheep. The dashed line shows the expected relationship if the two clearances were equal. The curved lines show the 95% confidence intervals for the calculated regression.

Discussion

Over the range of clearances studied Cl_{in} and Cl_{ecr} were very similar. However at high clearances Cl_{ecr} over-estimated Cl_{in}. Assuming that Cl_{in} actually measures GFR, this finding indicates secretion of creatinine by the renal tubule. Creatinine is known to be secreted by the proximal renal tubule in several species (Smith, 1951).

Similarly, at low Cl_{in}, Cl_{ecr} under-estimated Cl_{in}, suggesting reabsorption of creatinine. Reabsorption from the lower urinary tract occurs during exogenous creatinine infusion in dogs (Levinsky and Berliner, 1959) and at low urinary flow in man (Chesley, 1938). However since the studies reported herein did not demonstrate a significant correlation between Cl_{ecr}/Cl_{in} and urinary volume, it is unlikely that significant reabsorption of creatinine occurred from the lower urinary tract. In addition, significant reabsorption of creatinine within the nephron has only been demonstrated during exogenous creatinine infusion (Ladd, Liddle and Gagnon, 1956) and so is unlikely to account for these observations.

Alternatively, the findings may be a result of failure of the method to measure solely "true" creatinine. This seems the most likely explanation since it is known that adsorption of creatinine by Lloyd's reagent does not eliminate all "pseudo" creatinines in human urine (Narayanan *et al* 1972).

Despite the variation in Cl_{ecr}/Cl_{in}, the clearances of the two substances were strongly correlated. Errors introduced by this method are therefore probably not serious, especially in the detection of relative changes in GFR. Additionally, over the range of Cl_{in} 14-48 ml min^{-1}, including most of the observations, the

clearances of the two substances were not significantly different. The maximum deviation of the Cl_{ecr} versus Cl_{in} regression was 17%, scarcely greater than the observed within infusion coefficient of variation of Cl_{in} ($14.5 \pm 3.6\%$).

In conclusion, "true" creatinine apparently undergoes secretion and possibly reabsortion by the renal tubule of sheep. This error is probably due to non-specificity of the method with regard to a number of different constituents of plasma and does not constitute a serious source of error over the range of GFR normally encountered.

REFERENCES

Achilles, W., Scheidt, B., Hoppe, H. and Cumme, G.A. (1977) Eine ionenaustauschmethode zur bestimmung freier metallionen-konzentrationen, geeignet zur anwendung in biologischen flussigkeiten. Acta Biologica et Medica Germanica, **36**, 17-28.

Agus, Z.S., Chiu, P.J.S. and Goldberg, M. (1977) Regulation of urinary calcium excretion in the rat. American Journal of Physiology, **232**, F545-549.

Alcock, N.W. and Shils, M.E. (1969) Association of inorganic pyro-phosphatase activity with normal calcification of rat costal cartilage in vivo. Biochemical Journal, **112**, 505-510.

Alfrey, A.C. and Ibels, L.S. (1977) Role of phosphate and pyrophosphate in soft tissue calcification. Advances in Experimental Medicine and Biology, **103**, 187-193.

Altman, R.D., Johnstone, C.C., Khairi, M.R.A., Wellman, H., Serafini, A.N. and Sankey, R.R. (1973) Influence of disodium etidronate on clinical and laboratory manifestations of paget´s disease of bone. New England Journal of Medicine, **289**, 1379-1384.

Ardaillou, R., Vuagnat, P., Milhaud, G. and Richet, G. (1967) Effets de la thyrocalcitonine sur l´excrétion renále des phosphates, du calcium et des ions H^+ chez l´homme. Nephron, **4**, 298-314.

Barzel, U.S. and Jowsey, J. (1969) The effects of chronic acid and alkali administration on bone turnover in adult rats. Clinical Science, 36, 517-524.

Baum, P. and Czok, R. (1959) Enzymatische bestimmung von ionisierten magnesium im plasma. Biochemische Zeitschrift, 332, 121-130.

Bernstein, D., Kleeman, C.R., Cutler, R.E., Dowling, J.T. and Maxwell, M.H. (1962) Comparison of renal clearance of calcium during infusions of calcium chloride and calcium gluconate. Proceedings of the Society for Experimental Biology and Medicine, 110, 671-673.

Bird, J.S. and Maguire, M.E. (1978) The agonist-specific effect of magnesium ion on binding by B-adrenergic receptors in S49 lymphoma cells. Interaction of GTP and magnesium in adenylate cyclase activation. Journal of Biological Chemistry, 253, 8826-8834.

Bisaz, S., Felix, R. and Fleisch, H. (1975) Quantitative determination of ethane-1-hydroxy-1,1-diphosphonate in urine and plasma. Clinica Chimica Acta, 65, 299-307.

Bisaz, S., Jung, A. and Fleisch, H. (1978) Uptake by bone of pyrophosphate, diphosphonates and their technetium derivatives. Clinical Science and Molecular Medicine, 54, 265-272.

Bisaz, S., Russell, R.G.A. and Fleisch, H. (1968) Isolation of

inorganic pyrophosphate from bovine and human teeth. Archives of Oral Biology, **13**, 683-696.

Blood, D.C. and Henderson, J.A. (1974) Veterinary Medicine. 4th edition, London: Baillière Tindall.

Blood, D.C., Henderson, J.A. and Radostits, O.M. (1979) Veterinary Medicine. A Textbook of the Diseases of Cattle Sheep Pigs and Horses. 5th edition, London: Baillière Tindall.

Boyd, J. and Williamson, F.B. (1980) A physico-chemical study of heparin. Evidence for a calcium-induced co-operative conformational transition. Journal of Molecular Biology, **137**, 175-190.

Breen, M. and Freeman, S. (1961) The use of high centrifugal force in separating plasma calcium into its protein-bound and protein-free fractions. Clinica Chimica Acta, **6**, 181-187.

Bruce, H.M. (1950) The water requirements of laboratory animals. Journal of the Animal Technicians Association, **1**, 2-8.

Brunette, M. and Aras, M. (1971) A microinjection study of nephron permeability to calcium and magnesium. American Journal of Physiology, **221**, 1442-1448.

Brunette, M., Wen, S., Evanson, R.L. and Dirks, J.H. (1969) Micropuncture study of magnesium reabsorption in the proximal tubule of the dog. American Journal of Physiology, **216**, 1510-1516.

Brunette, M.G. and Crochet, M.E. (1975) Fluorometric method for the determination of magnesium in renal tubular fluid. Analytical Biochemistry, **65**, 79-88.

Brunette, M.G., Vigneault, N. and Carriere, S. (1978) Magnesium handling by the papilla of the young rat. Pfluegers Archiv, **373**, 229-235.

Buehrer, T.F. and Reitmeier, R.F. (1940) The inhibiting action of minute amounts of Na hexametaphosphate on the precipitation of calcium carbonate from ammoniacal solutions. II. Mechanism of the process with special reference to the formation of calcium carbonate crystals. Journal of Physical Chemistry, **44**, 552-574.

Burnell, J.M. (1971) Changes in bone sodium and carbonate in metabolic acidosis and alkalosis in the dog. Journal of Clinical Investigation, **50**, 327-331.

Buyske, D.A., Eisner, H.J. and Kelly, R.G. (1960) Concentration and persistance of tetracycline and chlortetracycline in bone. Journal of Pharmacology and Experimental Therapeutics, **130**, 150-156.

Care, A.D., Sherwood, L.M., Potts, J.T. and Aurbach, G.D. (1966) Perfusion of the isolated parathyroid gland of the goat and sheep. Nature, **209**, 55-57.

Carlyle, S.S (1975) Calcium Metabolism in Foetal Sheep. Ph.D. Thesis, University of Cambridge, pp. 20-21.

Carr, C.W. (1955) Competitive binding of calcium and magnesium with serum albumin. Proceedings of the Society for Experimental Biology and Medicine, 89, 546-549.

Carroll, R.L. and Irani, R.R. (1968) On the Δ H and Δ S of metal ion interaction with substituted methylene diphosphonates. Journal of Inorganic and Nuclear Chemistry, 30, 2971-2976.

Cartier, P. and Picard, J. (1955) La minéralisation du cartilage ossifiable. III. Le mecanisme de la réaction ATPasique du cartilage. Bulletin de la Société de Chimie Biologique, 37, 1159-1168.

Chanutin, A., Ludwig, S. and Masket, A.V. (1942) Studies on the calcium-protein relationship with the aid of the ultracentrifuge. I. Observations on calcium caesinate solutions. Journal of Biological Chemistry, 143, 737-751.

Chen, P.S. and Neuman, W.F. (1955a) Renal excretion of calcium by the dog. American Journal of Physiology, 180, 623-631.

Chen, P.S. and Neuman, W.F. (1955b) Renal reabsorption of calcium through its inhibition by various chemical agents. American Journal of Physiology, 180, 632-636.

Chenoweth, M.B. (1956) Chelation as a mechanism of pharmacological action. Pharmacological Reviews, 8, 57-87.

Chesley, L.C. (1938) Renal excretion at low urine volumes and the mechanism of oliguria. Journal of Clinical Investigation, **17**, 591-597.

Clapp, J.R., Rector, F.C. and Seldin, D.W. (1962) Effect of unreabsorbed anions on proximal and distal transtubular potentials in rats. American Journal of Physiology, **202**, 781-786.

Coburn, J.W., Massry, S.G. and Kleeman, C.R. (1970) The effect of calcium infusion on renal handling of magnesium with normal and reduced glomerular filtration rate. Nephron, **7**, 131-143.

Cohlan, S.Q., Bevelander, G. and Tiamsic, T. (1963) Growth inhibition of premature receiving tetracyclines: clinical and laboratory investigation. American Journal of Diseases of Children, **105**, 453-461.

Copeland, B.E. and Sunderman, F.W. (1952) XVIII. The magnesium binding property of the serum proteins. Journal of Biological Chemistry, **197**, 331-341.

Cummings, N.A., Kuff, E.L. and Sober, H.A. (1968) Examination of magnesium binding to serum proteins by ultracentrifugal analysis. Analytical Biochemistry, **22**, 108-116.

Davidson, W.D. and Sackner, M.A. (1963) Simplification of the anthrone method for the determination of inulin in clearance studies. Journal of Laboratory and Clinical Medicine, **62**, 351-356.

De Rouffignac, C., Morel, F., Moss, N. and Roinel, N. (1973) Micropuncture study of water and electrolyte movements along the loop of Henle in psammomys with special reference to magnesium, calcium and phosphorous. Pfluegers Archiv, **344**, 309-326.

Dekanič, D. (1979) The effect of chronic acidosis on bone composition in adult male and female rats. Archiv za Higijenu rada i Toksikologiju, **30**, 15-23.

Dibona, G.F. (1971) Effect of hypercalcaemia on renal tubular sodium handling in the rat. American Journal of Physiology, **220**, 49-53.

Dirks, J.H., Clapp, J.R. and Berliner, R.W. (1964) The protein concentration in the proximal tubule of the dog. Journal of Clinical Investigation, **43**, 916-921.

Donnan, F.G. (1924) The theory of membrane equilibria. Chemical Reviews, **1**, 73-90.

Dryerre, H. and Greig, J.R. (1925) Milk fever: Its possible association with derangements in the internal secretions. Veterinary Record, **5**, 225-231.

Dryerre, H. and Greig, J.R. (1928) Further studies in the etiology of milk fever. Veterinary Record, **8**, 721-728.

Dryerre, H. and Greig, J.R. (1935) The specific chemo-therapy of milk fever by the parenteral administration of calcium

borogluconate. Veterinary Record, **16**, 456-459.

Duncan, C.W., Huffman, C.F. and Robinson, C.S. (1935) Magnesium studies in calves. I. Tetany produced by a ration of milk or milk with various supplements. Journal of Biological Chemistry, **108**, 35-44.

Durbin, R.P. (1960) Osmotic flow of water across permeable cellulose membranes. Journal of General Physiology, **44**, 315-326.

Edwards, B.R., Sutton, R.A.L. and Dirks, J.H. (1974) Effect of calcium infusion on renal tubular reabsorption in the dog. American Journal of Physiology, **227**, 13-18.

Flatman, P.W. and Lew, V.L. (1980) Magnesium buffering in intact human red blood cells measured using the ionophore A23187. Journal of Physiology, **305**, 13-30.

Fleisch, H. (1975) Physico chemical and biological effects of pyrophosphate and diphosphonates. Colloques Internationeaux du Centre National de la Recherche Scientifique, **230**, Physico-chimie et cristallographie des apatites d›intérêt Biologique, 263-273.

Fleisch, H. (1980) Experimental basis for the clinical use of diphosphonates in paget´s disease of bone. Arthritis and Rheumatism, **23**, 1162-1170.

Fleisch, H. and Neuman, W.F. (1961) Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. American

Journal of Physiology, **200**, 1296-1300.

Fleisch, H., and Bisaz, S. (1962) Isolation from urine of pyrophosphate, a calcification inhibitor. American Journal of Physiology, **203**, 671-675.

Fleisch, H., and Bisaz, S. (1964) The inhibitory effect of pyrophosphate on calcium oxalate precipitation and its relation to urolithiasis. Experientia, **20**, 276-277.

Fleisch, H., Bisaz, S., Care, A.D., Mullbauer, R.C. and Russell, R.G.G. (1969) A review of the effects of phosphonates on calcium metabolism. In: Calcitonin 1969. Proceedings of the Second International Symposium, London. 409-417.

Fleisch, H., Russell, R.G.G. and Francis, M.D. (1969) Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture and in vivo. Science, **165**, 1262-1264.

Fleisch, H., Straumann, F., Schenk, R., Bisaz, S. and Allgower, M. (1966) Effect of condensed phosphates on calcification of chick embryo femurs in tissue culture. American Journal of Physiology, **211**, 821-825.

Fleisch, H.A., Russell, R.G.G., Bisaz, S., Mullbauer, R.C. and Williams, D.A. (1970) The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo. European Journal of Clinical Investigation, **1**, 12-18.

Foreman, H., Vier, M. and Magee, M. (1953) The metabolism of C^{14}-labelled ethylenediamine tetraacetic acid in the rat. Journal of Biological Chemistry, **203**, 1045-1053.

Forland, M., Pullman, T.N., Lavender, A.R. and Aho, I. (1966) The renal excretion of ethylenediaminetetraacetate in the dog. Journal of Pharmacology and Experimental Therapeutics, **153**, 142-147.

Francis, M.D. (1969) The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphate. Calcified Tissue Research, **3**, 151-162.

Frijlink, W.B., Bijvoet, O.L.M., te Velde, J. and Heynen, G. (1979) Treatment of pagets disease with (3-amino-1-hydroxypropylidene)-1,1-biphosphonate (APD). Lancet, **1**, 799-803.

Frizel, D.E., Malleson, A.G. and Marks, V. (1967) Measurement of plasma ionised calcium and magnesium by ion exchange strip. Clinica Chimica Acta, **16**, 45-56.

Frohlich, E.D., Scott, J.B. and Haddy, F.J. (1962) Effect of cations on resistance and responsiveness of renal and forelimb vascular beds. American Journal of Physiology, **203**, 583-587.

Gabbiani, G. (1966) Effect of phosphates on experimental skin calcinosis. Canadian Journal of Physiology and Pharmacology, **44**, 203-207.

Gasser, A.B., Morgan, D.B., Fleisch, H.A. and Richelle, L.J. (1972) The influence of two diphosphonates on calcium metabolism in the rat. Clinical Science, **43**, 31-45.

Gattinoni, L. and Samaja, M. (1979) Acid-base equilibrium in the blood of sheep. Experientia, **35**, 1347-1348.

Giebisch, G., Lauson, H.D. and Pitts, R.F. (1954) Renal excretion and volume of distribution of various dextrans. American Journal of Physiology, **178**, 168-176.

Girndt, J., Henning, H.V. and Delling, G. (1979) Correlation of calcium and acid base metabolism. Hormone and Metabolic Research, **11**, 587-588.

Givens, M.H. and Mendel, L.B. (1917) Studies in calcium and magnesium metabolism. I. The effects of base and acid. Journal of Biological Chemistry, **31**, 421-433.

Gomori, G. and Gulyas, E. (1944) Effect of parenterally administered citrate on the renal excretion of calcium. Proceedings of the Society for Experimental Biology and Medicine, **56**, 226-228.

Goodman, A.D., Lemann, J., Lennon, E.J. and Relman, A.S. (1965) Production, excretion, and net balance of fixed acid in patients with renal acidosis. Journal of Clinical Investigation, **44**, 495-506.

Goulding, A. and Broom, M.F. (1979) Effects of diphosphonate and

colchicine administration upon acid-base changes induced in rats by bilateral nephrectomy. Clinical Science, 57, 19-23.

Goulding, A., and Cameron, V. (1977) Changes in serum calcium concentration and urinary cyclic AMP excretion in rats after diphosphonate. Proceedings of the University of Otago Medical School, 55, 49-51.

Grabenstetter, R.J. and Cilley, W.A. (1971) Polynuclear complex formation in solutions of calcium ion and ethane-1-hydroxy-1,1-diphosphonic acid. I. Complexometric and pH titrations. Journal of Physical Chemistry, 75, 676-682.

Grabenstetter, R.J., Quimby, O.T. and Flautt, T.J. (1967) The acid dissociation constants of substituted methanediphosphonic acids: A correlation with P^{31} magnetic resonance chemical shift and with Taft σ. Journal of Physical Chemistry, 71, 4194-4202.

Greenberg, D.M. and Greenberg, M. (1931) I. Ultrafiltration of electrolytes from alkali caseinate solutions. Journal of Biological Chemistry, 94, 373-382.

Greenwald, I. (1941) The dissociation of calcium and magnesium carbonates and bicarbonates. Journal of Biological Chemistry, 141, 789-796.

Grollman, A.P., Walker, W.G., Harrison, H.C. and Harrison, H.E. (1963) Site of reabsorption of citrate and calcium in the renal tubule of the dog. American Journal of Physiology, 205, 697-701.

Harris, C.A., Burnatowska, M.A., Seely, J.F., Sutton, R.A.L., Quamme, G.A. and Dirks, J.H. (1979) Effect of parathyroid hormone on electrolyte transport in the hamster nephron. American Journal of Physiology, **236**, F342-348.

Heaton, F.W. (1967) The determination of ionized magnesium in serum and urine. Clinica Chimica Acta, **15**, 139-144.

Hill, L.F., Lumb, G.A., Mawer, E.B. and Stanbury, S.W. (1973) Indirect inhibition of the biosynthesis of 1,25-dihydroxychole-calciferol in rats treated with a diphosphonate. Clinical Science, **44**, 335-347.

Holland, J.F., Danielson, E. and Sahagian-Edwards, A. (1953) Use of ethylene diamine tetra acetic acid in hypercalcaemic patients. Proceedings of the Society for Experimental Biology and Medicine, **84**, 359-364.

Holley, D.C. and Evans, J.W. (1977) Determination of total and ultrafiltrable calcium and magnesium in normal equine serum. American Journal of Veterinary Research, **38**, 259-262.

Howard, P.J., Wilde, W.S. and Malvin, R.L. (1959) Localization of renal calcium transport; effect of calcium loads and of gluconate anion on water, sodium and potassium. American Journal of Physiology, **197**, 337-341.

Hughes, S.P.F., Davies, D.R., Bassingthwaighte, J.B., Knox, F.G.

and Kelly, P.J. (1977) Bone extraction and blood clearance of diphosphonate in the dog. American Journal of Physiology, **232**, H341-347.

Ibsen, K.H. and Urist, M.R. (1962) Complexes of calcium and magnesium with oxytetracycline. Proceedings of the Society for Experimental Biology and Medicine, **109**, 797-801.

Irving, L. and Chute, A.L. (1932) The participation of the carbonates of bone in the neutralization of ingested acid. Journal of Cellular and Comparative Physiology, **2**, 157-176.

Izutsu, K.T. and Felton, S.P. (1972) Plasma calcium assay, with the use of the jellyfish protein, aequorin, as a reagent. Clinical Chemistry, **18**, 77-79.

Izutsu, K.T., Felton, S.P., Siegel, I.A., Nicholls, J.I, Crawford, J., McGough, J. and Yoda, W.T. (1974) An investigation of the ionic calcium assay based on the jellyfish protein aequorin. Analytical Biochemistry, **58**, 479-484.

Jaffe, H.L., Bodansky, A. and Chandler, J.P. (1932) Ammonium chloride decalcification as modified by calcium intake; the relationship between generalized osteoporosis and ostitis fibrosa. Journal of Experimental Medicine, **56**, 823-834.

Janicki, R.H. and Argyris, T.S. (1969) Kidney growth and adaptation of phosphate-dependent glutaminase in the mouse. American Journal of Physiology, **217**, 1389-1395.

Johnson, L.A. and Seven, M.J. (1961) Proceedings of a conference on biological aspects of metal binding. Federation Proceedings, **20**, Supplement 10, 1-273.

Jowsey, J., Holley, K.E. and Linman, J.W. (1970) Effect of sodium etidronate in adult cats. Journal of Laboratory and Clinical Medicine, **76**, 126-133.

Jowsey, J., Riggs, B.L., Kelly, P.J., Hoffman, D.L. and Bordier, P. (1971) The treatment of osteoporosis with disodium ethane-1-hydroxy-1,1-diphosphonate. Journal of Laboratory and Clinical Medicine, **78**, 574-584.

Kaye, M. (1973) Effect of a diphosphonate on uremic hyperparathyroidism. Nephron, **10**, 188-194.

Kelly, H.G., Cross, H.C., Turton, M.R. and Hatcher, J.D. (1960) Renal and cardiovascular effects induced by intravenous infusion of magnesium sulphate. Canadian Medical Association Journal, **82**, 866-871.

Keměny, A., Boldizăr, H. and Pethes, G. (1961) The distribution of cations in plasma and cerebrospinal fluid following infusion of solutions of salts of sodium, potassium, magnesium and calcium. Journal of Neurochemistry, **7**, 218-227.

Kent, P., Bunce, S.C., Bailey, R.A. and Aikens, D.A. (1974) Interference by polyamines in the measurement of magnesium ion at

physiological pH with the divalent cation-selective electrode. Analytical Biochemistry, **62**, 75-80.

Kielland, J. (1937) Individual activity coefficients of ions in aqueous solutions. Journal of the American Chemical Society, **59**, 1675-1678.

Killen, A.D., Grogan, E.L., Gower, R.E. and Collins, H.A. (1971) Response of canine plasma ionized calcium to the rapid infusion of acid-citrate-dextrose (ACD) solutions. Surgery, **70**, 736-743.

Kim, Y.S. and Padilla, G.M. (1978) Determination of free calcium ion concentrations with an ion selective electrode in the presence of chelating agents in comparison with calculated values. Analytical Biochemistry, **89**, 521-528.

King, W.R., Francis, M.D. and Michael, W.R. (1971) Effect of disodium ethane-1-hydroxy-1,1-diphosphonate on bone formation. Clinical Orthopaedics and Related Research, **78**, 251-270.

Kleeman, C.R., Bernstein, D., Dowling, J.T. and Maxwell, M.H. (1960) The effect of parathyroidectomy and administration of partially purified parathyroid extract on the renal clearance of calcium and phosphorous in the dog. Acta Endocrinologica Supplementum, **51**, 493.

date

Kleeman, C.R., Rockney, R.E. and Maxwell, M.H. The effect of parathyroid extract on the renal clearance of diffusible calcium. Journal of Clinical Investigation, **37**, 907.

Knudsen, E. (1959) Renal clearance studies on the horse. I. Inulin, endogenous creatinine and urea. Acta Veterinaria Scandinavica, 1, 52-66.

Ladd, M., Liddle, L. and Gagnon, A. (1956) Renal excretion of inulin, creatinine and ferrocyanide, at normal and reduced clearance levels in the dog. American Journal of Physiology, 184, 505-514.

Ladensen, J.H. and Bowers, G.N. (1973) Free calcium in serum. I. Determination with the ion specific electrode, and factors affecting the results. Clinical Chemistry, 19, 565-574.

Lamb, A.R. and Evvard, J.M. (1919) The acid-base balance in animal nutrition. II. Metabolism studies on the effect of certain organic and mineral acids on swine. Journal of Biological Chemistry, 37, 329-342.

Larsson, L. and Ohman, S. (1980) Nova-2 ionized calcium analyser compared with the Orion SS-20. Clinical Chemistry, 26, 1761-1762.

Lassiter, W.E., Gottschalk, C.W. and Mylle, M. (1963) Micropunture study of renal tubular reabsorption of calcium in normal rodents. American Journal of Physiology, 204, 771-775.

Lauson, H.D. (1951) Sources of error in plasma creatinine determinations. Journal of Applied Physiology, 4, 227-244.

Le Grimellec, C., Poujeol, P. and de Rouffignac, C. (1975) ^{3}H inulin and electrolyte concentrations in Bowman's capsule in rat kidney. Pfluegers Archiv, 354, 117-131.

Lemann, J., Lennon, E.J., Goodman, A.D., Litzow, J.R. and Relman, A.S. (1965) The net balance of acid in subjects given large loads of acid or alkali. Journal of Clinical Investigation, 44, 507-517.

Lemann, J., Litzow, J.R. and Lennon, E.J. (1966) The effects of chronic acid loads in normal man: further evidence for the participation of bone mineral in the defense against chronic metabolic acidosis. Journal of Clinical Investigation, 45, 1608-1614.

Lemkes, H.H.P.J., Reitsma, P.H., Frijilink, W., Verlinden-Ooms, H. and Bijvoet, O.L.M. (1977) A new diphosphonate: dissociation between effects on cells and mineral in rats and a preliminary trial in paget's disease. Advances in Experimental Medicine and Biology, 103, 459-469.

Levinsky, N.G. and Berliner, R.W. (1959) Changes in composition of the urine in ureter and bladder at low urine flow. American Journal of Physiology, 196, 549-553.

Levinsky, N.G. and Levy, M. (1973) In: Handbook of Physiology, Section 8, pp. 103-117, Ed. Orloff, J. and Berliner, R.W., Washington D.C.: American Physiological Society.

Levinson, W.E. (1980) Chelating substances. Antibiotics and

Chemotherapy, **27**, 288-306.

Levitt, M.F., Halpern, M.H., Polimero, D.P., Sweet, A.Y. and Gribetz, D. (1958) The effect of abrupt changes in plasma calcium concentrations on renal function and electrolyte excretion in man and monkey. Journal of Clinical Investigation, **37**, 294-305.

Lindenhayn, K., Trzenschik, K., Buhler, G. and Wegner, G. (1982) On the action of prednisolone and ethane-1-hydroxy-1,1-diphosphonate (EHDP) on rabbit bone. Experimental Pathology, **21**, 157-164.

Lins, L. (1979) Renal function in hypercalcaemic dogs during hydropenia and during saline infusions. Acta Physiologica Scandinavica, **106**, 177-186.

Ludwig, S., Chanutin, A. and Masket, A.V. (1942) Studies on the calcium-protein relationship with the aid of the ultracentrifuge. II. Observations on serum. Journal of Biological Chemistry, **143**, 753-761.

MacIntyre, I., Boss, S. and Troughton, V.A. (1963) Parathyroid hormone and magnesium homeostasis. Nature, **198**, 1058-1060.

Macpherson, H.T. and Stewart, J. (1938) XIV. Investigations of the nature of calcium borogluconate. Biochemical Journal, **32**, 76-78.

Martell, A.E. (1960) Chelation: stability and selectivity. Annals of the New York Academy of Sciences, **88**, 284-292.

Martell, A.E. and Calvin, M. (1952) Chemistry of the metal chelate compounds. New York: Prentice Hall.

Massry, S.G., Coburn, J.W. and Kleeman, C.R. (1969) Renal handling of magnesium in the dog. American Journal of Physiology, **216**, 1460-1467.

Massry, S.G., Coburn, J.W., Chapman, L.W. and Kleeman, C.R. (1967) The interrelationship between serum calcium, sodium excretion, and the parathyroids on renal handling of calcium. Journal of Clinical Investigation, **46**, 1092-1093.

May, P.M., Linder, P.W. and Williams, D.R. (1977) Computer simulation of metal-ion equilibria in biofluids: models for the low molecular-weight complex distribution of calcium (II), magnesium (II), manganese (II), iron (III), copper (II), zinc (II), and lead (II) ions in human blood plasma. Journal of the Chemical Society, **38**, 588-595.

McLean, F.C. and Hastings, A.B. (1934a) A biological method for the estimation of calcium ion concentration. Journal of Biological Chemistry, **107**, 337-350.

McLean, F.C. and Hastings, A.B. (1934b) The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. Journal of Biological Chemistry, **108**, 285-322.

Mendel, L.B. and Benedict, S.R. (1909a) The paths of excretion for inorganic compounds.-IV. The excretion of magnesium. American

Journal of Physiology, 25, 1-22.

Mendel, L.B. and Benedict, S.R. (1909b) The paths of excretion for inorganic compounds.-V. The excretion of calcium. American Journal of Physiology, 25, 23-33.

Meyer, J.L. (1974) Formation constants for interaction of citrate with calcium and magnesium ions. Analytical Biochemistry, 62, 295-300.

Michael, W.R., King, W.R. and Francis, M.D. (1971) Effectiveness of diphosphonates in preventing "osteoporosis" of disuse in the rat. Clinical Orthopaedics and Related Research, 78, 271-276.

Michael, W.R., King, W.R. and Wakim, J.M. (1972) Metabolism of disodium ethane-1-hydroxy-1,1-diphosphonate (disodium etidronate) in the rat, rabbit, dog and monkey. Toxicology and Applied Pharmacology, 21, 503-515.

Miura, M., Otani, S., Kodama, M. and Shinagwa, K. (1962) Effect of several diphosphonates on the crystallization and crystal habit of strontium sulphate. Journal of Physical Chemistry, 66, 252-253.

Moeller, T. and Horwitz, E.P. (1960) Chelation. In: Mineral Metabolism, vol. 1A, Chapter 4, pp. 101-118, Ed. Comar, C.L. and Bronner, F., London: Academic Press Inc. (London) Ltd.

Moore, E.W. (1970) Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes. Journal of

Clinical Investigation, **49**, 318-334.

Morel, F., Roinel, N. and Le Grimellec, C. (1969) Electron probe analysis of tubular fluid composition. Nephron, 6, 350-364.

Morgan, D.B., Monod, A., Russell, R.G.G. and Fleisch, H. (1973) Influence of dichloromethylene diphosphonate (Cl_2MDP) and calcitonin on bone resorption, lactate production, and phosphatase and pyrophosphatase content of mouse calvaria treated with parathyroid hormone in vitro. Calcified Tissue Research, **13**, 287-294.

Mulbauer, R.C., Russell, R.G.G., Williams, D.A. and Fleisch, H. (1971) The effects of diphosphonates, polyphosphates and calcitonin on "immobilization osteoporosis" in rats. European Journal of Clinical Investigation, 1, 336-344.

Murayama, Y., Morel, F. and Le Grimellec, C. (1972) Phosphate, calcium and magnesium transfers in proximal tubules and loops of Henle, as measured by single nephron microperfusion experiments in the rat. Pfluegers Archiv, **333**, 1-16.

Narayanan, S. and Appleton, H.D. (1972) Specificity of accepted procedures for urine creatinine. Clinical Chemistry, **18**, 270-274.

Neuhausen, B.S. and Marshall, E.K. (1922) An electrochemical study of the condition of several electrolytes in the blood. Journal of Biological Chemistry, **53**, 365-372.

Neuman, W.F. and Neuman, M.W. (1958) The Chemical Dynamics of Bone Mineral, Chicago: University of Chicago Press.

Neuman, W.F., Morrow, P.E., Toribara, T.Y., Casarett, L.J., Mulryan, B.J. and Hodge, H.C. (1956) Evidence for complex ion formation in the calcium bicarbonate system. Journal of Biological Chemistry, **219**, 551-555.

Newell, G.K. and Beauchene, R.E. (1975) Effects of dietary calcium level, acid stress, and age on renal, serum, and bone responses of rats. Journal of Nutrition, **105**, 1039-1047.

Nixon, G.A, Buehler, E.V. and Newmann E.A. (1972) Preliminary safety assessment of disodium etidronate as an additive to experimental oral hygiene products. Toxicology and Applied Pharmacology, **22**, 661-671.

Nordbo, R. (1939) Bestimmung der magnesiumionenkonzentration im ultrafiltrat von blutserum. Skandinavisches Archiv fuer Physiologie, **81**, 265-268.

Owen, J.A, Iggo, B., Scandrett, F.J. and Stewart, C.P. (1954) The determination of creatinine in plasma or serum, and in urine; a critical examination. Biochemical Journal, **58**, 426-437.

Parfitt, A.M. (1969) Study of parathyroid function in man by EDTA infusion. Journal of Clinical Endocrinology and Metabolism, **29**, 569-580.

Parsons, J.A. and Robinson, C.J. (1971) Calcium shift into bone causing transient hypocalcaemia after injection of parathyroid hormone. Nature, **230**, 581-582.

Pedersen, K.O. (1970) Determination of calcium fractions of serum. II. Investigation of calcium ion activity and stability of important calcium complexes by an improved semimicro method. Scandinavian Journal of Clinical and Laboratory Investigation, **25**, 199-208.

Pedersen, K.O. (1971a) The effect of bicarbonate, pCO_2 and pH on serum calcium fractions. Scandinavian Journal of Clinical and Laboratory Investigation, **27**, 145-150.

Pedersen, K.O. (1971b) Multiple small sample ultrafiltration without special apparatus. Scandinavian Journal of Clinical and Laboratory Investigation, **28**, 57-59.

Pedersen, K.O. (1972a) Binding of calcium to serum albumin. II. Effect of pH via competitive hydrogen and calcium ion binding to the imidazole groups of albumin. Scandinavian Journal of Clinical and Laboratory Investigation, **29**, 75-83.

Pederson, K.O. (1972b) Binding of calcium to serum albumin. III. Influence of ionic strength and ionic medium. Scandinavian Journal of Clinical and Laboratory Investigation, **29**, 427-432.

Pederson, K.O. (1972c) Binding of calcium to serum albumin. IV. Effect of temperature and thermodynamics of calcium albumin

interaction. Scandinavian Journal of Clinical and Laboratory Investigation, 30, 89-94.

Pederson, K.O. (1972d) Protein bound calcium in human serum. Quantitative examination of binding and its variables by a molecular binding model and clinical chemical implications for measurement of ionized calcium. Scandinavian Journal of Clinical and Laboratory Investigation, 30, 321-329.

Perkins, H.R. and Walker, P.G. (1958) The occurence of pyrophosphate in bone. Journal of Bone and Joint Surgery, 40B, 333-339.

Pors Nielsen, S. (1970) Abolition of magnesium-induced hypocalcaemia by acute thyro-parathyroidectomy in the cat. Acta Endocrinologica, 64, 150-158.

Pors Nielsen, S., Falch Christiansen, T., Hartling, O. and Trap-Jensen, J. (1977) Increase in serum ionized calcium during exercise. Clinical Science and Molecular Medicine, 53, 579-586.

Pullman, T.N., Lavender, A.R. and Foreland, M. (1963) Synthetic chelating agents in clinical medicine. Annual Review of Medicine, 14, 175-194.

Quamme, G.A. and Dirks, J.H. (1980) Intraluminal and contraluminal magnesium on magnesium and calcium transfer in the rat nephron. American Journal of Physiology, 238, F187-198.

Ramberg, C.F., Mayer, G.P., Kronfeld, D.S., Aurbach, G.D., Sherwood, L.M. and Potts, J.T. (1967) Plasma calcium and parathyroid hormone responses to EDTA infusion in the cow. American Journal of Physiology, **213**, 878-882.

Rashid, M., Khairi, A., and Johnston, C. (1977) Treatment of paget´s disease of bone (osteitis deformans) with sodium etidronate (EHDP). Clinical Orthopaedics and Related Research, **127**, 94-104.

Rawson, A.J. and Sunderman, F.W. (1948) Studies in serum electrolytes. XV. The calcium-binding property of the serum proteins (Multiple myeloma, lymphogranuloma venereum and sarcoidosis). Journal of Clinical Investigation, **27**, 82-90.

Recker, R.R. and Saville, P.D. (1973) Intestinal absorption of disodium ethane-1-hydroxy-1,1-diphosphonate (disodium etidronate) using a deconvolution tecnique. Toxicology and Applied Pharmacology, **24**, 580-589.

Regna, P.P., Solomons, I.A., Murai, K., Timreck, A.E., Brunings, K.J. and Lazier, W.A. (1951) The isolation and general properties of terramycin and terramycin salts. Journal of the American Chemical Society, **73**, 4211-4215.

Reitemeier, R.F. and Ayers, A.D. (1947) Calcium ion activities in supersaturated solutions stabilized by sodium metaphosphate as determined by clay membrane electrodes. Journal of the American Chemical Society, **69**, 2759-2761.

Relman, A.S., Lennon, E.J. and Lemann, J. (1961) Endogenous production of fixed acid and the measurement of the net balance of acid in normal subjects. Journal of Clinical Investigation, **40**, 1621-1630.

Renkin, E.M. (1954) Filtration, diffusion, and molecular sieving through porous cellulose membranes. Journal of General Physiology, **38**, 225-243.

Robertson, W.G. and Fleisch, H. (1970) The effect of imidodiphosphate (P-N-P) on the precipitation and dissolution of calcium phosphate in vitro. Biochimica et Biophysica Acta, **222**, 677-680.

Robertson, W.G. and Peacock, M. (1968) New techniques for the separation and measurement of the calcium fractions of normal human serum. Clinica Chimica Acta, **20**, 315-326.

Robertson, W.G., Morgan, D.B., Fleisch, H. and Francis, M.D. (1972) The effects of diphosphonates on the exchangeable and non-exchangeable calcium and phosphate of hydroxy apatite. Biochemica et Biophysica Acta, **261**, 517-525.

Rocha, A.S., Magaldi, J.B. and Kokko, J.P. (1977) Calcium and phosphate transport in isolated segments of rabbit henle's loop. Journal of Clinical Investigation, **59**, 975-983.

Rodan, G.A., Lieberman, U.A., Paran, M. and Anbar, M. (1967) Lack of physicochemical equilibrium between blood and bone calcium in

the isolated perfused dog limb. Israel Journal of Medical Sciences, 3, 702-713.

Rona, P. and Takahashi, D. (1911) Uber das verhalten des calciums im serum und uber den gehalt der blutkorperchen an calcium. Biochemische Zeitschrift, 31, 336-344.

Rose, G.A. (1957) Determination of the ionized and ultrafiltrable calcium of normal human plasma. Clinica Chimica Acta, 2, 227-236.

Rosenblum, I.Y. (1974) The effects of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) on bone and serum chemistry in rabbits. Calcified Tissue Research, 16, 145-152.

Ross, J.W. (1967) Calcium-selective electrode with liquid ion exchanger. Science, 156, 1378-1379.

Roux, R. and Barlet, J.P. (1980) Ammonium chloride in the prophylaxis of parturient hypocalcaemia. Annales de Recherches Veterinaires, 11, 195-200.

Rubin. M. and Lindenblad, G.E. (1956-7) Chelating agents in the study of renal absorption of alkaline earth cations. Annals of the New York Academy of Sciences, 64, 337-342.

Russell, J.E., Termine, J.D. and Avioli, L.V. (1975) Experimental renal osteodystrophy. The response to 25-hydroxycholecalciferol and dichloromethylene diphosphonate therapy. Journal of Clinical Investigation, 56, 548-554.

Russell, R.G.G. and Fleisch, H. (1975) Pyrophosphate and diphosphonates in skeletal metabolism. Clinical Orthopaedics and Related Research, **108**, 241-263.

Russell, R.G.G., Bisaz, S., Donath, A., Morgan, D.B. and Fleisch, H. (1971) Inorganic pyrophosphate in plasma in normal persons and in patients with hypophosphatasia, osteogenesis imperfecta, and other disorders of bone. Journal of Clinical Investigation, **50**, 961-969.

Samiy, A.H.E., Brown, J.L., Globus, D.L., Kessler, R.H. and Thompson, D.D. (1960) Interrelation between renal transport systems of magnesium and calcium. American Journal of Physiology, **198**, 599-602.

Schibler, D., Russell, R.G.G. and Fleisch, H. (1968) Inhibition by pyrophosphate and polyphosphate of aortic calcification induced by vitamin D_3 in rats. Clinical Science, **35**, 363-372.

Schmidt-Nielsen, B., Osaki, H., Murdaugh, H.V. and O'Dell, R. (1958) Renal regulation of urea excretion in sheep. American Journal of Physiology, **194**, 221-228.

Schwartz, H.D. (1975) Serum ionized calcium by electrodes: new technology and methodology. Clinica Chimica Acta, **64**, 227-239.

Schwartzenbach, G. (1957) Complexometric Titrations (Translated by H. Irving), London: Methuen and Co. Ltd. New York: Interscience.

Scott, P.J. and Hurley, P.J. (1968) Demonstration of individual variation in constancy of 24-hour urinary creatinine excretion. Clinica Chimica Acta, **21**, 411-414.

Shakhmatova, E.I., Kabachnik, M.I., Medved′ T. Ya. and Natochin, Yu. V. (1982) Ability of diphosphonates to bind calcium and their effect on osmotic permeability of the frog bladder wall. Byulleten′ Eksperimental′noi Biologii i Meditsiny, **93**, 71-74.

Shannon, J.A. (1937) Excretion of inulin, creatinine, xylose and urea in the sheep. Proceedings of the Society for Experimental Biology and Medicine, **37**, 379-380.

Shareghi, G.R. and Agus, Z.S. (1979) Magnesium transport in the rabbit cortical thick ascending limb (CTAL). Clinical Research, **27**, 430A.

Sherwood, L.M, Potts, J.T., Care, A.D., Mayer, G.P, and Aurbach, G.D. (1966) Evaluation by radioimmunoassay of factors controlling the secretion of parathyroid hormone. Intravenous infusion of calcium and ethylenediamine tetraacetic acid in the cow and goat. Nature, **209**, 52-55.

Siggaard-Andersen, O. (1961) Sampling and storing of blood for determination of acid-base status. Scandinavian Journal of Clinical and Laboratory Investigation, **13**, 196-204.

Singer, F.R., Woodhouse, N.J.Y., Parkinson, D.K. and Joplin, G.F.

(1969) Some acute effects of administered porcine calcitonin in man. Clinical Science, 37, 181-190.

Smith, H.W. (1951) The Kidney. Structure and Function in Health and Disease, pp. 182-194, New York: Oxford University Press.

Soffer, A. and Toribara, T. (1961) Changes in serum and spinal fluid calcium effected by disodium ethylenediaminetetraacetate. Journal of Laboratory and Clinical Medicine, 58, 542-547.

Soncin, E. (1953) Fenomeni di interferenza tra elettroliti e antibiotici III. Ione magnesio e aureomicina, terramicin, claramfenicolo. Archives Internationales de Pharmacodynamie et de Thérapie, 94, 346-352.

Soulier, J.P. and Crosnier, J. (1958) Nouvelle méthode de dosage du calcium ionisé utilisant un système coagulant. I. Technique. La Presse Medicale, 66, 617-619.

Speich, M., Bosquet, B. and Nicolas, G. (1981) Reference values for ionized, complexed, and protein bound plasma magnesium in men and women. Clinical Chemistry, 27, 246-248.

Spencer, H., Vankinscott, V., Lewin, I. and Lazlo, D. (1952) Removal of calcium in man by ethylenediamine tetra-acetic acid. A metabolic study. Journal of Clinical Investigation, 31, 1023-1027.

Stacy, B.D. (1969) Augmented renal excretion of calcium and magnesium in sheep after feeding. Quarterly Journal of Experimental

Physiology, **54**, 1-10.

Stacy, B.D. and Wilson, B.W. (1970) Acidosis and hypercalciuria: renal mechanisms affecting calcium, magnesium and sodium excretion in the sheep. Journal of Physiology, **210**, 549-564.

Subramanian, G., McAfee, J.G., Blair, R.J., Mehter, A. and Connor, T. (1972) ^{99m}Tc-EHDP: A potential radiopharmaceutical for skeletal imaging. Journal of Nuclear Medicine, **13**, 947-950.

Sutton, R.A.L., Wong, N.L.M. and Dirks, J.H. (1976) Effects of parathyroid hormone on sodium and calcium transport in the dog nephron. Clinical Science and Molecular Medicine, **51**, 345-351.

Sutton, R.A.L., Wong, N.L.M. and Dirks, J.H. (1979) Effects of metabolic acidosis and alkalosis on sodium and calcium transport in the dog kidney. Kidney International, **15**, 520-533.

Talmage, R.V. and Kraintz, F.W. (1954) Progressive changes in renal phosphate and calcium excretion in rats following parathyroidectomy or parathyroid administration. Proceedings of the Society for Experimental Biology and Medicine, **87**, 263-267.

Tofaletti, J., Savory, J. and Gitelman, H.J. (1977) Use of gel filtration to examine the distribution of calcium among serum proteins. Clinical Chemistry, **23**, 2306-2310.

Treschel, U., Schenk, R., Bonjour, J.P., Russell, R.G.G. and Fleisch, H. (1977) Relation between bone mineralization, calcium

absorption, and plasma calcium in phosphonate treated rats. American Journal of Physiology, **232**, E298-305.

Tschimber, H. and Tschimber, C. (1924) Tecnique de l'ultrafiltration du plasma; détermination du pH et du calcium, du magnésium, du sodium et du phosphore dans l'ultrafiltrat. Comptes Rendus des Séances de la Société de Biologie, **91**, 592-593.

Vagg, M.J. and Payne, J.M. (1970) The effect of ammonium chloride induced acidosis on calcium metabolism in ruminants. British Veterinary Journal, **126**, 531-537.

Varley, H., Gowenlock, A.H. and Bell, M. (1980) Practical Clinical Chemistry, 5th edn., vol. I, London: W. Heinmann Medical Books Ltd.

Wacker, W.E.C. and Vallee, B.L. (1964) In: Mineral Metabolism, vol. 2A, page 290, Ed. Comar, C.L. and Bronner, F., London: Academic Press (London) Ltd.

Walser, M. (1960) Determination of free magnesium ions in body fluids. Improved methods for free calcium ions, total calcium and total magnesium. Analytical Chemistry, **32**, 711-717.

Walser, M. (1961a) Ion association. VI. Interactions between calcium, magnesium, inorganic phosphate, citrate and protein in normal human plasma. Journal of Clinical Investigation, **40**, 723-730.

Walser, M. (1961b) Ion association. V. Dissociation constants for

complexes of citrate with sodium, potassium, calcium, and magnesium ions. Journal of Physical Chemistry, **65**, 159-161.

Walser, M. (1961c) Ion association. VII. Dependence of calciuresis on natriuresis during sulphate infusion. American Journal of Physiology, **201**, 769-773.

Walser, M. (1969) Renal excretion of alkaline earths. In: Mineral Metabolism, vol. 3, pp. 235-320, Ed. Comar, C.L. and Bronner, F., New York and London: Academic Press.

Walton, R.J., Russell, R.G.G. and Smith, R. (1975) Changes in the renal and extrarenal handling of phosphate induced by disodium etidronate (EHDP) in man. Clinical Science and Molecular Medicine, **49**, 45-56.

Warwick, I.S. (1966) Urine collection apparatus for male horses. Journal of Science Technology, **12**, 181-182.

Weinberg, E.D. (1958) Enhancement of bacitracin by the metallic ions of group IIB. Antibiotics Annual, 924-929.

White, R.D., Goldsmith, R.S. Rodriguez, R., Moffit, E.A. and Pluth, J.R. (1976) Plasma ionic calcium levels following injection of chloride, gluconate and glucaheptate salts of calcium. Journal of Thoracic and Cardiovascular Surgery, **71**, 609-613.

Widrow, S.H. and Levinsky, N.G. (1962) The effect of parathyroid extract on renal tubular calcium reabsorption in the dog. Journal

of Clinical Investigation, **41**, 2151-2159.

Wiers, B.H. (1971) Polynuclear complex formation in solutions of calcium ion and ethane-1-hydroxy-1,1-diphoshonic acid. II. Light scattering, sedimentation, mobility and dialysis measurements. Journal of Physical Chemistry, **75**, 682-687.

Windsor, E. and Cronheim, G.E. (1961) Gastro-intestinal absorption of heparin and synthetic heparinoids. Nature, **190**, 263-264.

Wolf, A.V. and Ball, S.M. (1949) Effect of intravenous calcium salts on renal excretion in the dog. American Journal of Physiology, **158**, 205-217.

Wolf, A.V. and Ball, S.M. (1950) Effect of intravenous sodium sulphate on renal excretion in the dog. American Journal of Physiology, **160**, 353-360.

Yarrington, J.T., Capen, C.C., Black, H.E., Re, R., Nagode, L.A. and Geho, W.B. (1977a) Effect of dichloromethylene diphosphonate on calcium homeostatic mechanisms in pregnant cows. American Journal of Pathology, **87**, 615-632.

Yarrington, J.T., Capen, C.C., Black, H.E., Re, R., Potts, J.T. and Geho, W.B. (1977b) Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) on the ultrastructure of parathyroid glands and plasma immunoreactive parathyroid hormone in pregnant cows fed a low calcium diet. Laboratory Investigation, **36**, 402-412.

Yendt, E.R., Connor, T.B. and Howard, J.E. (1955) In vitro calcification of rachitic rat cartilage in normal and pathological human sera with some observations on the pathogenesis of renal rickets. Bulletin of the Johns Hopkins Hospital, **96**, 1-19.

Young, M.K. and Raisz, L.G. (1952) An anthrone procedure for the determination of inulin in biological fluids. Proceedings of the Society for Experimental Biology and Medicine, **80**, 771-774.