Rarity of kidney stones in South Africa's black population: Studies of urinary macromolecules, crystal matrix extract containing osteopontin, and bone turnover markers in urine and serum from black and white subjects as a key to understanding this paradox

by

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ABSTRACT

The work described in this thesis was undertaken to investigate physicochemical, biochemical and physiological factors contributing towards the rarity of kidney stone disease in the South African black population. Healthy, age-matched male subjects from the black and white population groups were recruited for this purpose. In several of the studies, subjects followed a standardized diet and were required to provide 24 hour urine collections. These were analyzed for sodium, potassium, calcium, oxalate, uric acid, citrate, chloride, magnesium, phosphate, sulphate and creatinine using standard laboratory techniques. Urine composition values were used as input data for the calculation of relative supersaturation (RS) values for calcium oxalate (CaOx), calcium phosphate (CaP, or brushite) and uric acid (UA) using the computer programme EQUIL and for the calculation of the Tiselius Risk Index (TRI). CaOx crystallization experiments were performed. These included CaOx metastable limit (MSL) and BONN Risk Index (BRI) determinations, particle formation kinetics, ^{14}C-oxalate crystal deposition kinetics and CaOx crystal aggregation and nucleation inhibition. Crystallization experiments were also supplemented with scanning electron microscopy (SEM) and zeta potential measurements. Urine compositions, crystallization data and physicochemical risk indices were analyzed statisitically using ANOVA.

Several different investigations were undertaken. These included crystallization experiments involving urinary macromolecules from both race groups, crystal matrix extract isolation (with osteopontin as its major component) from both race groups and its testing for inhibitory capacity in ultrafiltered urine from both race groups. Similar crystallization experiments were conducted with commercially available osteopontin. In addition, a comprehensive trial was conducted in which the ingestion of three sodium salts (sodium chloride, sodium bicarbonate and sodium citrate) was investigated for their effects on urinary risk factors of CaOx stone formation and for their effects on bone turnover markers in urine and in serum.

For the biochemical isolation of crystal matrix extract, COD-CME was precipitated in urine from both black and white subjects. The proteins included in COD-CME were detected using sodium dodecyl sulfate polyacryalamide gel electrophoresis (SDS-PAGE). Western Blotting was used for semi-quantitative analysis of OPN.

For the trial involving different sodium salts, four experimental protocols were investigated. The four protocols included low NaCl (3 g/day), high NaCl (12 g/day), sodium bicarbonate (6 g/day) and sodium citrate in the form of Citro-Soda (16 g/day). A Latin Square Design was followed for the random assignment of participants to sequences of protocols.

The studies on macromolecules showed that those in the urine of black subjects were more potent inhibitors of CaOx crystal deposition and aggregation than those in the urine of white subjects. Isolation and characterization of the crystal matrix extract in COD crystals confirmed that osteopontin is the main intracrystalline protein in the extract. The crystallization experiments performed on the crystal matrix extract isolated from the urine of both race groups demonstrated that the extract isolated from the urine of black subjects was a superior inhibitor of CaOx deposition, growth and aggregation. Crystallization studies performed on commercially available osteopontin in urine from black and white subjects showed that this protein is a more effective inhibitor of CaOx crystal deposition, growth and nucleation in the urine from the former group compared to that from the latter.

The studies on supplemental sodium salts demonstrated that the two race groups respond differently to lithogenic and anti-lithogenic dietary challenges. High NaCl protocol resulted in a favourable and counter-intuitive significant decrease in free unbound calcium in samples from black subjects whereas no such change was observed in white subjects. Supplemental sodium bicarbonate and sodium citrate induced favourable decreases in urinary total calcium, urinary ionized calcium, BRI, RS of CaOx, RS of uric acid and RS of brushite, and a favourable increase in urinary citrate and pH in both groups. Interestingly and more importantly, these factors were more prominent in samples from black subjects than those from white subjects.

Bone turnover measurements showed that urinary deoxypyridinoline (DPD) levels were lower while serum osteocalcin (OC) levels were higher in blacks than in whites at baseline, but these differences were not statistically significant. Smaller increases in urinary DPD levels after high NaCl and sodium bicarbonate and corresponding bigger increases in serum osteocalcin (OC) levels after these protocols (and sodium citrate) in black subjects than in white subjects indicate less bone resorption and higher bone formation, respectively, in the former group.

The results presented in this thesis have provided convincing evidence that in the context of CaOx kidney stone formation, several physicochemical, biochemical and physiological factors are different in black and white South African subjects and that these factors are more effective in the former group with respect to providing protective mechanisms against CaOx kidney stone formation.

List of Abbreviations

ANOVA	Analysis of variance
BAP	Bone alkaline phosphatase
BC	Concentrate from black subjects
BNTHP	Tamm-Horsfall Protein isolated from black normal subjects
BP	Prefiltrate from black subjects
BSFTHP	Tamm-Horsfall Protein isolated from black stone formers
BSA	Bovine serum albumin
BUF	Ultrafiltered urine from black subjects
C	Concentrate
CaOx	Calcium oxalate
CaP	Calcium Phosphate
CME	Crystal matrix extract
COM	Calcium oxalate monohydrate
COD	Calcium oxalate dihydrate
COD-CME	Calcium oxalate dihydrate-crystal matrix extract
COT	Calcium oxalate trihydrate
CS	Chondroitin sulphate
Ctrl	Control
CTX	COOH-terminal telopeptide
DPD	Deoxypyiridinoline
DS	Dermatan sulphate
GAGS	Glycosaminoglycans
HP	Heparan sulphate
ITI	Inter-α-Trypsin Inhibitor
KS	Keratan sulphate
MSL	Metastable limit
Na_2Ox	Sodium oxalate
NC	Nephrocalcin
NTX	NH_2-terminal telopeptide
N_{IA}	Aggregation inhibition
N_{IN}	Nucleation inhibition

OD	Optical Density
OPN	Osteopontin
P	Prefiltrate
PYD	Pyridinoline
RS	Relative Supersaturation
SEM	Scanning Electron Microscopy
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
THP	Tamm-Horsfall Protein
TALP	Total alkaline phosphatase
UF	Ultrafiltrate
UP	Uropontin
UPTF1	Urinary prothrombin fragment-1
UA	Uric acid
WC	Concentrate from white subjects
WNTHP	Tamm-Horsfall Protein isolated from white normal subjects
WP	Prefiltrate from white subjects
WSFTHP	Tamm-Horsfall Protein isolated from white stone formers
WUF	Ultrafiltered urine from white subjects
XRD	X-ray powder diffraction

DIET protocol Abbreviations

L	Low NaCl
H	High NaCl
C	Sodium Bicarbonate
D	Sodium Citrate

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CHAPTER ONE

INTRODUCTION

Urolithiasis (kidney stone disease) occurs throughout the world afflicting about 8 – 20 % of the population in western societies (Robertson and Hughes 1994; Balla et al. 1998; Pak 1998; Kamoun et al. 1999; Bennani et al. 2000; Ramello et al. 2000; Bill and Meyers 2001; Decoster et al. 2002; Morton et al. 2002; Bulo et al. 2004; Parmar 2004; Trinchieri 2006). The most common component of these stones is calcium oxalate (CaOx). Of great interest is that the incidence of urolithiasis in the South African black population is extremely rare while in the white population it is similar to that which occurs in other western societies (Muskat 1951; Modlin 1967; Whalley et al. 1998). This provides basic scientists with a unique opportunity to study the two population groups with a view to identifying and characterizing the multiple factors which cause the one group to be stone-free and the other group to be stone-prone. Numerous physicochemical, biochemical and renal handling mechanisms are likely to be involved in explaining this phenomenon. The present thesis describes investigations into some of these processes.

1.1 KIDNEY STONES AND THEIR CHEMICAL COMPOSITION

Urolithiasis is one of mankind's oldest diseases and continues to pose a universal health problem (Robertson and Peacock 1982; Ryall 1993; Tiselius 2000, 2003; Khan and Kok 2004). Kidney stones are hard, solid pellets that form in the urinary tract and are the cause of one of the most painful ailments. In addition to pain, kidney stones can also lead to renal failure (Koga et al. 1991; Ramello et al. 2000; Bihl and Meyers 2001; Pietrow et al. 2006; Trinchieri 2006). In many cases they are very small and can pass through the urinary tract without any problems and discomfort (Robertson and Peacock 1972; Hallson and Rose 1976; Finlayson et al. 1990; Khan and Kok 2004). However, if a stone (even a small one) becomes lodged and blocks the flow of urine, excruciating pain may result. Occasionally salts form in urine from various ions such as calcium and oxalate that nucleate to form calcium oxalate (CaOx) crystals which build up on the inner surfaces of the kidney (Robertson et al. 1978; Coe and Parks 1988; Kumar et al. 2003). These salts can become concentrated under certain circumstances e.g. if the volume of urine is significantly reduced; or if extremely high amounts of crystal-forming ions are present. When concentration levels exceed

supersaturation levels, salts can precipitate and form crystals which could later grow and aggregate to form a stone.

Generally there are four types of stones, namely calcium, uric acid, struvite and cystine stones (Finlayson 1977; Finlayson 1978; Khan and Hackett 1987; Coe and Parks 1988). Calcium stones are the most common of all kidney stones, and occur in the form of calcium oxalate (CaOx) or calcium phosphate (CaP) (Khan et al. 1979; Nancollas 1983; Tiselius et al. 1995; Aggarwal et al. 2000; Kok 2002; Coe et al. 2005). About 70 % of calcium-containing stones are caused by hypercalciuria (abnormally high calcium in the urine).

CaOx stones

CaOx stones are either composed of pure calcium oxalate or a mixture of calcium oxalate and calcium phosphate (Gibson 1974; Iida et al. 2003; Worcester et al. 2006). Approximately 85 % of calcium stones are composed of pure CaOx. Because of this predominance, CaOx stones comprise the basis for the origin of this thesis. CaOx is an insoluble complex that forms when calcium is chemically bound to oxalate. Pure CaOx containing stones exist either in monohydrate (COM) or dihydrate (COD) crystal form or in rare cases trihydrate (COT) (Herring 1962; Streit et al. 1998; Grases et al. 1998; Bithelis et al. 2004; El-Shall et al. 2004). COM is the most common form of CaOx crystals in renal stones. This type of stone is due to hypercalciuria and/or hyperoxaluria (excess oxalate in urine), and in most cases are idiopathic (indicating the absence of any clinical cause of the disease) (Marangella et al. 2000; Coe et al. 2005). The pathogenesis of idiopathic CaOx stone formation is multifactorial. Most of these factors are epidemiological, physicochemical and dietary and each of them will be discussed in detail in the following pages.

CaP stones

About 10 % of calcium stones are composed of CaP (Mandel and Mandel 1989). This type of stone is relatively uncommon and occurs in patients with metabolic or hormonal disorders such as hyperparathyroidism (excessive parathyroid hormone levels) (Buck 1990; Hess and Jaeger 1993) and renal tubular acidosis (inherited condition in which the kidneys are unable to excrete acid) (Carlisle et al. 1991). High urinary pH and low urinary citrate are the driving forces for the formation of these stones (Pak et al. 1978). CaP stones exist in two forms:

hydroxyapatite (Calcium hydroxyl phosphate; $Ca_5(PO_4)_3(OH)$) and brushite (calcium hydrogen phosphate $CaHPO_4.2H_2O$). Hydroxyapatite is the most thermodynamically stable CaP crystal phase and is the major component in mixed CaOx/CaP stones (Tiselius and Larsson 1992; Baumann et al. 2001). Brushite has a very high recurrence rate in patients with this stone type compared to hydroxyapatite (Tiselius 2000). Brushite stones are very physically resistant structures which are difficult to break with shock waves. However both CaP stone forms are soluble in acidic media (Klee et al. 1991; Evan et al. 2005).

Uric Acid stones

About 5 to 10 % of stones are made up of uric acid, which is a breakdown product of purine (Ramello et al. 2000; Coe et al. 2005). Uric acid is produced in the liver and enters the bloodstream, where most of it passes into the kidneys and is eliminated in urine. This type of stone is mainly due to hyperuricosuria which is caused by high levels of uric acid and low urinary pH levels (Pak et al. 2002; Sakhaee et al. 2002; Abate et al. 2004; Zarse et al. 2004).

Struvite stones

Struvite stones (magnesium ammonium phosphate) are almost always caused by urinary tract infections due to bacteria (e.g. *Proteus, Pseudomonas* etc.) that secrete certain enzymes. These enzymes degrade urea to ammonia and carbonate. This in turn, raises urine concentrations of ammonia and causes the urine to be more alkaline. This alkalinity subsequently promotes the formation of struvite stones (Ramello et al. 2000).

Cystine stones

Cystine stones develop from genetic defects that cause abnormal transport of amino acids in the kidney and gastrointestinal system leading to a build-up of cystine (cystinuria) which is one of these amino acids. The tendency to form these stones is inherited and they are marked by rapid growth and recurrence, which, if not treated promptly, can eventually lead to kidney failure (Font-Llitjós et al. 2005). About 2 % of this type of stone is found in adults and up to 8% in children and is reported to occur in between 0.07 and 0.40 % of the population (Font-Llitjós et al. 2005). Cystine is highly soluble at pH > 7.2 and this is even more pronounced in the presence of urinary macromolecules (Nakagawa et al. 2000) and with increasing ionic strength (Pak and Fuller 1983). Cystine is an oxidized dimeric form of cysteine (an alpha

amino acid containing sulphur that is found in most proteins especially in keratin) molecules joined together by a weak disulfide bond (Aslaksena et al. 2006). Cystine has a chemical formula of $C_6H_{12}N_2O_4S_2$ and that of cysteine is $C_3H_7NO_2S$ (Aslaksena et al. 2006).

1.2 EPIDEMIOLOGICAL RISK FACTORS OF STONE FORMATION

The incidence of kidney stones varies throughout the world and may be correlated to age, gender, race, geography, climate and diet (Scott 1985; Beukes et al. 1987; Sakhaee et al. 1987; Rodgers and Spector1981; Ramello et al. 2000).

1.2.1 Age and Gender

Kidney stones affect about 15 % of men and 5 % of women by the time they are 30 to 50 years old (Blacklock 1982; Hesse et al. 1986; Iguchi et al. 1999). Twice as many males are afflicted by this disease than females (Blacklock 1982; Hesse et al. 1986; Hess and Kok 1996; Iguchi et al. 1999; Heller et al. 2002). The higher incidence in men compared to women may be due to the fact that men generally excrete more calcium, oxalate and uric acid than women (Robertson et al. 1980; Blacklock 1982). Generally, the incidence of kidney stones in children is very low and this can be accounted for by their relatively low excretion of calcium and high excretion of polyanion inhibitors (Robertson et al. 1980; Robertson and Peacock 1983). These inhibitors consist of glycosaminoglycans (GAGS) and non-polymerised proteins which have the ability to retard aggregation and growth of CaOx crystals (Robertson and Peacock 1983).

1.2.2 Race

The incidence of kidney stones has been reported to be higher in whites than blacks, irrespective of the geographical and environmental area (Robertson and Peacock 1981; Whalley et al. 1998; Ramello et al. 2000). These reports have been supported by a very recent study conducted by Maloney et al. (2005) in which 1,141 stone patients from the same geographic region (North Carolina, USA) participated. Out of these patients, only 9 % were non-white. This study reported a higher prevalence of hypercalciuria in whites compared to the non-white group (67 % vs. 25 % respectively). A low incidence of kidney stones is also reported in other race groups such as Eskimos, Black Americans and Aborigines (Widdowson and McCance 1970; Bateson 1997). In USA and Brazil, the ratio of white to black stone-formers is reported to be four-to-one (Ramello et al. 2000).

In South Africa, kidney stone formation in the black population is extremely rare, while in the white group it occurs with a frequency rate of about 10 – 15 % (Modlin 1967; Beukes et al. 1987; Meyers et al. 1994; Whalley et al. 1998; Pinnock et al. 2004). This intriguing phenomenon provides the overall motivation for the present PhD project. The multiple aspects associated with this unique situation will be discussed later in this chapter.

1.2.3 Geography

In the USA, the highest occurrence of kidney stones occurs in the south while the lowest occurs in the west (Boyce and Strawcutter 1956; Prince and Scardino 1960). It is suggested that the higher incidence may be due to a higher rate of hypertension in the south and certain dietary habits, particularly lower intake of magnesium and low use of calcium supplements (Boyce and Strawcutter 1956). In the South Eastern Stone Belt (South Eastern United States), the incidence of stone disease has been reported to be higher than elsewhere in the USA (Boyce and Strawcutter 1956; Prince and Scardino 1960; Blacklock 1982). This high incidence could be due to high atmospheric temperatures which lead to dehydration through sweating. This trend is also reported in Australia (Blacklock 1982), Europe (Blacklock 1982; Schwille and Hermann 1987; Decoster et al. 2002), Asia (Balla et al. 1998) and Saudi Arabia (Robertson 1999) and is even more pronounced during the hot summer time than in winter (Pak 1991; Balla et al. 1998; Kamoun et al. 1999; Bennani et al. 2000; Decoster et al. 2002). In some regions of Africa e.g. Nigeria (Esho 1978; Olapade-Olaopa et al. 2004), Southern Sudan (Kambal et al. 1981), Tanzania (Mkony et al. 1991) and South Africa (with racial differences (Modlin 1967; Whalley et al. 1998; Pinnock et al. 2004)) the occurrence of stones is extremely rare. However, there are other African countries such as Cameroon (Angwafo et al. 2000), Algeria (Harrache et al. 1997) and Egypt (Loutfi et al. 1978) in which endemic bladder stones occur in the black population. In Northern Sudan (Ibrahim 1978; Kambal et al. 1981) and Kenya (Clennon et al. 2004; King et al. 2004) a prevalence of CaOx upper urinary tract is reported.

1.2.4 Climate

People who are exposed to hot sunny climates (e.g. people in the geographic areas discussed above and those who live in desserts) are generally susceptible to CaOx urolithiasis (Robertson et al. 1974; Hallson and Rose 1976; Blacklock 1982). Hot sunny climates adversely influence some of the main urinary risk factors (Robertson and Peacock 1983) such

as low urinary volume and pH which result from excessive loss of liquid through sweating (Schwille and Hermann 1987). Another risk factors which is adversely affected in hot climates is urinary calcium excretion. An increase in urinary calcium can occur as a result of prolonged exposure to ultra-violet radiation (vitamin D) which subsequently leads to increased intestinal calcium absorption (Robertson et al. 1980; Broadus et al. 1984).

1.2.5 Diet

Diet is a highly important epidemiological factor which can significantly influence urine composition. The multiple aspects of diet in the pathogenesis of urolithiasis are fully discussed in Section 1.6.

1.2.6 Genetic factors

CaOx stone disease has been reported to be a polygenic disorder (Goodman et al. 1995). This is supported by studies by Curhan et al. (1997) and Mente et al. (2006) which reported an increased risk of stone formation in first-degree relatives of kidney stone formers. Studies in South Africa have also revealed that cystine stones (inherited stones) and struvite stones are very rare in the black population compared to their white counterparts (Abdel Goad and Bereckzy 2003; Pinnock et al. 2004).

1.3 MECHANISM OF STONE FORMATION

There are several major fundamental physicochemical mechanisms involved in stone formation. These include supersaturation, nucleation, crystal growth and aggregation (Hess and Kok 1996; Khan and Kok 2004).

1.3.1 Supersaturation

Supersaturation is the first and the most essential step in crystallization which eventually leads to stone formation. It is the thermodynamic driving force that occurs when there is a phase change from liquid to solid and it is a representation of excess free energy between the two phases (Finlayson 1978; Hess and Kok 1996; Kavanagh 2006 (a)). This driving force or free energy (ΔG) is given by the equation:

$$\Delta G = RT \ln (A / Aeq)$$

where R is the gas constant, T is the temperature, A is the activity of the unionized salt in the supersaturated solution and Aeq is the activity of the solution at equilibrium (Finlayson 1978; Hess and Kok 1996; Kavanagh 2006 (a), (b)). The supersaturation ratio, (A / Aeq), is referred to as the relative supersaturation (RS). When RS < 1, the solution is said to be undersaturated and any stone forming salts that are present can freely dissolve. When RS = 1, the solution is saturated. In such a case, salts which are already present will not dissolve while new salts will not form. At RS > 1, the solution is supersaturated. In this situation, existing crystals may grow (Finlayson 1978; Hess and Kok 1996, Coe et al. 2005). Researchers have reported that urine from stone formers tends to be more supersaturated than those of non-stone formers (Robertson et al. 1968; Marangella et al. 1985; Coe and Favus 1986; Kok and Papapoulos 1993). Contrary to this, other researchers (Robertson et al. 1968; Ryall 1993; Hess and Kok 1996; Kavanagh 2006 (a)) have reported that some people who have never formed a stone nonetheless pass highly supersaturated urine. Supersaturation, therefore is insufficient to explain the occurrence and formation of stones (Robertson et al. 1968; Kok and Papapoulos 1993; Ryall 1993).

1.3.2 Crystal Nucleation

Nucleation is the initial kinetic step that allows phase transformation from liquid into a solid phase in a supersaturated solution (Finlayson 1978; Brown and Purich 1992; Kok and Khan 1994). The initial step in this process is the coalescence of stone forming salts in solution into loose clusters that may increase in size due to the addition of new clusters (Boskey 1981; Kok and Khan 1994). Gradually, these clusters become crystal embryos with all the already developing nuclei (Kok and Khan 1994).

Aqueous solutions of CaOx can remain crystal-free even when RS values lie between 80 and 100. Such solutions are referred to as being in the zone of metastability (Hess and Kok 1996). When the solution surpasses the upper limit of metastability, the solution rapidly becomes unstable, and new crystals are spontaneously formed. This process of spontaneous formation of new crystals is called homogeneous nucleation (Hess and Kok 1996). The supersaturation that is required for homogeneous nucleation is much higher than the equilibrium saturation but can be reduced by foreign particles which act as nucleation catalysts. Heterogenous nucleation occurs in the presence of foreign impurities and is thought to be the most common and frequent process in stone formation as most stones consist of mixtures of more than one type of crystals and a mixture of proteins (Hess and Kok 1996).

Many urinary macromolecules act as heterogeneous nucleators of stone formation, in whose presence crystal precipitation takes place at lower RS values (Hess and Kok 1996).

1.3.3 Crystal Growth

The incorporation of crystal components into an already formed CaOx crystal nucleus is termed crystal growth (Hess and Kok 1996). This occurs once a crystal nucleus has achieved its critical size and RS always remains above 1 (Kavanagh 2006 (a)). In order for crystal growth to occur, the newly formed crystal in the urine has to migrate through the solution and attach to the surface of the existing crystals. This process is called adsorption (Brown and Purich 1992; Hess and Kok 1996).

Precipitation of CaOx crystals might not pose a problem as long as the formed crystals can pass freely through the urinary tract. The normal transit through the kidney is not long enough for crystals to grow to size range in which can be trapped in the renal tubules. However, once these crystals are retained and become too big to pass freely through the renal tubules they disturb the urinary flow. Crystal growth is therefore considered important in stone formation because, unless the crystals grow to a very sufficient size, they will not form a stone (Finlayson et al. 1984; Kok and Khan 1994). The rate at which crystal growth takes place is also dependent on supersaturation (Kavanagh 2006 (a), (b)).

1.3.4 Crystal Aggregation

Crystal aggregation is the process by which several particles in solution stick together to form larger particles (Hess and Kok 1996). Aggregation of larger particles is energetically favoured and can therefore occur in all states of saturation (Kok and Khan 1994; Hess and Kok 1996). It is achieved through several basic forces, which have opposing effects (Finlayson 1978; Robertson et al. 1981; Hess 1991; Kok and Khan 1994). The attractive van der Waals force favours particle aggregation and increases strongly when interparticle distances are very small. Zeta potential is the repulsive electrostatic force that favours disaggregation of crystalline particles. Curreri et al. (1979) reported that in water, COM crystals have a zeta potential of +20 mV; whereas in urine zeta potential becomes negative due to the adsorption of negatively charged urinary molecules such as citrate and macromolecules (Finlayson et al. 1984). These negative charges set up repulsive forces which retard aggregation.

Several researchers believe that crystal aggregation is the most important factor for kidney stone formation (Meyer et al. 1971; Blomen 1982; Finlayson and Reid 1978; Iwata et al. 1985). This is due to the fact that CaOx crystal growth alone may be too slow to produce clinically significant particles within the time that urine passes through the renal tubules, whereas aggregation occurs within seconds. In addition to this, microscopic and ultrastructural analyses of kidney stones have revealed that stones are highly aggregated structures (Meyer et al. 1971; Iwata et al. 1985). Interestingly, urine from stone-formers contains more aggregated crystals whereas that from healthy subjects has less aggregated particles (Kok and Khan 1994).

1.4 INHIBITORS OF CALCIUM OXALATE CRYSTALLIZATION

The process of stone formation discussed in the previous pages is counteracted by inhibitors of CaOx crystallization (Hess and Kok 1996).

Inhibitors of calcium oxalate crystallization are classified into two categories: macro-molecules and micromolecules. Macromolecules such as glycosyaminoglycans (GAGS) and proteins such as nephrocalcin (NC), Tamm-Horsfall protein (THP), albumin, urinary prothrombin fragment-1 (UPTF1), bikunin, crystal matrix extract protein (CME) and osteopontin (also called uropontin; OPN) are discussed below. Micromolecules (citrate, magnesium and pyrophosphate) will be discussed under urinary risk factors (Section 1.5).

1.4.1 Glycosyaminoglycans (GAGS)

GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit. The disaccharide unit consists of an N-acetyl-hexosamine (amino sugar) and a hexose or hexuronic acid, either or both of which may be sulfated. Both constituents are linked together by a glycoside bond. The combination of the sulfate group and the carboxylate groups of the uronic acid residues gives them a very high density of negative charge (Nishio et al. 1985; Roberts and Resnick 1986).

The well-known urinary GAGS are heparan sulphate (HS), chondroitin sulfate (CS), dermatan sulphate (DS), keratan sulphate (KS), and non-sulphated hyaluronic acid (HA). Some of these are found in urinary crystals and stones but it is not clear whether this selective inclusion is due to differences in excretion rate or calcium affinity for calcium salt crystals

(Khan and Kok 2004). GAGS generally inhibit CaOx crystal growth, aggregation and nucleation (Koide et al. 1981; Sallis et al. 1981; Ryall and Marshall 1984; Breslau et al. 1994). HS has been reported to have the highest inhibitory potential followed by CS and HA (Sidhu et al. 1986; Khan et al. 1988; Kok et al. 1988; Ryall et al. 2001; Lieske et al. 1995; Verkoelen et al. 1996; Khan et al. 2000).

There are contradicting reports on the excretion levels of GAGS by stone-formers and healthy individuals. Some studies report a decreased excretion by stone formers (Conte et al. 1989; Michellacci et al 1989; Shum and Gohel 1993; Akcay et al. 1999) whereas others show an equal excretion by both stone formers and non-stone formers (Samuell 1981; Fellström et al. 1986; Akinci et al. 1991; Trinchieri et al. 1992; Harangi et al. 1996). However, Erturk et al. (2002) found that GAGS from stone formers had an increased nucleation promoting activity.

1.4.2 Urinary proteins

Crystal Matrix Protein Extract (CME)

The discovery of an organic component of kidney stones called the crystal matrix dates back to 1684 when Von Hyde discovered that stones have an organic framework composed mainly of selectively incorporated proteins generally characterized by high glutamic and aspartic acid content and γ-carboxyglutamic acid (Butt 1956; Binette et al. 1996). In general, urinary stones contain about 2-5 % organic matrix (Boyce and Garvey 1956; Stacholy and Goldberg 1985) and reports by Boyce (1968) have demonstrated that 67 % of this matrix is protein.

In 1987, Khan and Hackett (1993) had demonstrated that the organic matrix becomes closely incorporated in the crystals during the early stages of crystal formation. Prior to this report, Morse and Resnick (1988, 1989) had identified proteins incorporated in CaOx crystals induced in human urine and they found that this process is a selective phenomenon.

The theory of urinary protein inclusion and adsorption into CaOx crystals was also tested by Khan and co-workers using transmission electron microscopy (Khan et al. 1988). Their results showed that proteins have a strong affinity for CaOx crystals. They also discovered that adsorption of anionic proteins was sensitive to calcium ion concentration

whereas cationic protein adsorption depended upon the oxalate ion concentration (Khan et al. 1988).

The effect of CME on CaOx crystallization has been tested in undiluted urine (Koide et al. 1981; Doyle et al. 1995; Sorensen et al. 1995). From these studies it was reported that CME inhibits CaOx crystal growth and aggregation.

Researchers have investigated and identified several proteins associated with CME. These include THP (Doyle et al. 1991); albumin (Ryall et al. 1991; Cerini et al. 1999), nephrocalcin (Hess et al. 1989; Worcester 1996), UPTF1 (Ryall et al. 1997), inter-α-inhibitor (Atmani et al. 1996; Dean et al. 2000) and OPN (Worcester 1996; Asplin et al. 1998; Ryall et al. 2005). Studies involving these individual proteins (rather than the composite mixture described in the previous paragraphs) are discussed below.

Tamm-Horsfall Protein (THP)

Tamm-Horsfall Protein (THP) is the most abundant protein in normal human urine with a molecular weight of approximately 80 kDa. Its concentration in human urine varies between 20 to 100 mg/day (Khan and Kok 2004). THP exists in a monomeric state but tends to self-aggregate to a polymeric form in the presence of increased concentrations of free calcium, magnesium, sodium and at low pH. Inhibition or promotion of CaOx crystallization is influenced by its state of aggregation. The aggregated form of THP is a poor inhibitor in this regard compared to the non-aggregated entity (Hess et al. 1989).

Aggregation studies by Hess et al. (1989) have demonstrated that THP is a potent inhibitor of crystal aggregation by 50 % at urinary concentrations of 1 X 10^{-8} mol/L and this increases up 90 % at concentrations of 5 X 10^{-8} mol/L. THP isolated from urine from normal subjects has been shown not to be significantly different in terms of concentration from the one isolated from stone formers but structurally the latter contains less carbohydrates (sialic acid) (Hess et al. 1995 (a)). The less sialic acid content in THP from stone formers makes it a poor inhibitor of CaOx crystallization (Hess et al. 1995 (a)).

Albumin

Albumin is the second most abundant protein in urine (Boyce and Garvey 1956; Fraij 1989). It has been detected in the crystal matrix (Atmani et al. 1998, 2002) derived from whole human urine and also from urinary stones (Boyce 1968; Fraij 1989). The daily excretion of albumin by healthy humans ranges from 1.6 - 34.2 mg/day. In solution albumin is known to exist either in monomeric or polymeric forms and in metastable CaOx solutions both forms promote nucleation of CaOx crystals (Cerini et al. 1999). On the other hand, it has been shown that albumin binds to CaOx as well as to uric acid crystals and inhibits CaOx crystal aggregation and growth (Worcester 1994; Dussol et al. 1995; Hess et al. 1995 (a); Grover et al. 1998). According to a study by Cerini et al. (1999), nucleation by albumin leads exclusively to COD crystal formation which is less adherent to renal epithelial cells and this might be a protective feature by albumin against COM crystal formation.

Urinary Prothrombin Fragment-1 (UPTF1)

A study conducted by Doyle et al. (1991) showed that UPTF1 is the principal protein of the organic matrix of CaOx crystals induced in human urine. It was originally called crystal matrix protein (CMP) because it was the first protein to be identified in the organic crystal matrix. Studies conducted later with CMP showed that its N-terminus was identical to that of UPTF1, leading to the conclusion that it was the urinary form of prothrombin F1 (Stapleton et al. 1993). UPTF1 has a molecular weight of 31 kDa and when subjected to a series of cleavages it releases fragments which include fragment 1 (F1) and fragment 2 (F2) or a combination of both F1 + F2. The daily urinary excretion of UPTF1 is 13.4 nmol/L (Bezeau et al. 1984). In pregnant women it increases to 47.2 nmol/day. UPTF1 is located in the ascending limb of the loop of Henle in the human kidney and is present in greater quantities in stone formers compared to healthy controls (Stapleton et al. 2000). It also occurs in CaOx stones (Hoyer 1994; Stapleton et al. 2000).

The inhibitory capability of UPTF1 rests in the presence of 10 γ-carboxyglutamate (Gla) residues which contribute to the inhibition of CaOx crystal growth and aggregation by virtue of their calcium-binding affinity (Grover and Ryall 2002; Webber et al. 2002). The protein contains about 154 amino acids and 23 % are glutamic and aspartic acids and 10 of the carbonic acids are γ-carboxylated (Grover and Ryall 2002) which also play a role in its calcium affirnity.

Bikunin

Bikunin, also known as Inter-α-Trypsin Inhibitor (ITI) is a member of the I α I family which is a group of plasma protease inhibitors (Hochstrasser et al. 1976; Enghild et al. 1989; Salier et al. 1996) composed of a combination of heavy chains with molecular weights of 60 kDa (H1), 70 kDa (H2) and 90 kDa (H3) which are covalently linked to a light chain with a molecular weight of 35 - 45 kDa. Numerous investigators have reported different concentrations of bikunin in normal human urine: 0.225 - 0.650 μg/ml (Dean et al. 2000), 5.01 ± 0.91 μg/ml (Médétognon-Benissan et al. 1999), 10.13 ± 1.13 μg/ml and 6.72 ± 0.93 μg/ml in healthy men and women respectively (Nishio et al. 2001), 4.82 ± 2.46 mg/day and 3.86 ± 1.35 mg/day in healthy men and women respectively (Usui et al. 1984) and 17.5 mg/day (Kobayashi et al. 1998). It is even more increased in the urine of patients with renal disease (Suzuki et al. 1999; Khan and Kok 2004). Contrary to this, (Médétognon-Benissan et al. 1999) have reported that the concentrations are higher in healthy controls (5 mg/L) than in stone formers (2.54 mg/L). Of interest is the study by Atmani et al. (1994) who demonstrated that bikunin isolated from kidney stone patients contains less sialic acid than that purified from the urine of healthy subjects and that it inhibits crystallization to a lesser extent. These results were subsequently supported by a study conducted by Médétognon-Benissan (1999).

Many investigators have shown that ITI is an inhibitor of CaOx crystallization (Atmani et al. 1993 (a), (b), 1994; Kobayashi et al. 1998; Atmani and Khan 1999; Médétognon-Benissan 1999; Dean et al. 2000). Bikunin has also been shown to inhibit CaOx crystal adhesion to renal epithelial cells at minimum concentrations of 10 ng/ml and completely blocked it at 200 ng/ml (Ebusino et al. 1999).

Nephrocalcin (NC)

Nephrocalcin is a glycoprotein with a molecular weight of 14 kDa (Coe et al. 1994). However, it has a tendency to polymerize into dimers, trimers and tetramers with varying molecular weights of 23-30, 45-48, 60-68 kDa respectively (Nakagawa et al. 1983, 1985, 1987). Several studies have shown that NC inhibits growth, aggregation and nucleation of CaOx crystals at urinary concentrations of 10^{-6} to 10^{-8} mol/L (Nakagawa et al., 1983, 1985; Asplin et al. 1991; Coe et al. 1991; Tiselius et al. 1995; Worcester 1996). This inhibition may be attributed to electrostatic repulsive forces created when NC binds to CaOx crystals and the presence of glutamic and aspartic acid which have a high affinity for calcium

(Nakagawa et al. 1983). NC also inhibits binding of COM crystals to epithelial cells (Lieske and Toback 1993). Nakagawa et al. (1983, 1985) reported that NC isolated from the urine of stone formers did not contain the Gla residues and thus showed less inhibitory activity towards COM crystal formation.

Osteopontin (OPN)

Osteopontin (OPN), also known as uropontin (when isolated from urine), is a multifunctional noncollagenous phosphoprotein that was originally isolated from mineralized bone matrix. It has a molecular weight which varies from 44 to 75 kDa (Malyankar et al. 1997). This huge variation in molecular weight is assumed to be due to differences in glycosylation and phosphorylation (Denhardt and Guo 1993). It is synthesized within the kidney and is detected in the thick and thin ascending limb of the loop of Henle and distal convoluted tubules (Kleinman et al. 2004). OPN exists as a monomer but may also form aggregates which appear at very high molecular weights of 120 - 150 kDa (Johnson-Tardieu et al. 2000, Webber et al. 2003). It undergoes considerable post-translational modification and is easily phosphorylated and glycosylated (Denhardt and Guo 1993; Mazzali et al. 2002).

OPN contains about 50 % of glutamic and aspartic acid residues which easily bind to calcium (Franzen and Heinegard 1985; Chen et al. 1992; Denhardt and Guo 1993; Hunter et al. 1996) and as such, may have an inhibitory role in CaOx crystallization as proposed by Kohri et al. (1990). On the other hand, some researchers have proposed that OPN promotes stone formation, by supporting attachment of CaOx crystals to tubular cell membranes (Kohri et al. 1993; Yamate et al. 1998) via its arginine-glycine-aspartic acid sequence (Kohri et al. 1990). This sequence is referred to as RGD, where each symbol specifically represents the respective amino acids. Contrary to this, some researchers have reported that OPN blocks COM crystal adhesion to renal tubular cells (Lieske et al. 1999; Kumar et al. 2003). The differences in the potency of OPN from stone formers and healthy controls in CaOx crystallization is still unclear (Kleinman et al. 2004).

The mean OPN excretion for normal humans varies between 2.4 and 3.7 mg/day (Chalko et al. 1992) but it has also been reported as being as low as 1.9 μg/ml (Min et al. 1998). Studies by Nishio et al. (2001) reveal that stone formers excrete less OPN than stone free people, presumably due to the incorporation of OPN in the growing stone crystals. In their respective studies, Worcester et al. (1995) and Asplin et al. (1998) have demonstrated

that OPN is a potent inhibitor of CaOx crystal nucleation, growth and aggregation. On the other hand Wesson and co-workers have reported that OPN promotes crystallization of COD in preference to COM (Wesson et al. 1998), but they make the point that crystals of the former type have a lower affinity for renal epithelium cells than COM crystals, thereby leading to the prevention or minimization of kidney stone formation.

In a very recent study investigating the selective inclusion of proteins in CaOx crystals, OPN was found to be the main intracrystalline protein in COD crystals (Ryall et al. 2005). This contradicts the report by Hoyer (1994) that OPN was found in COM stones (with very low concentrations in COD stones).

General Comment

As mentioned earlier, kidney stones are extremely rare in the South African black population. The Kidney Stone Research Laboratory at the University of Cape Town (of which the author of this thesis is a member) has investigated several urinary proteins in this context. These include UPTF1 (Durrbaum et al. 2001; Webber et al. 2002), Tamm-Horsfall Protein (Craig et al. 1999, 2000, 2001), albumin (Rodgers et al. 2006) and bikunin (Mabizela 2006). Details of these investigations are provided in section 1.8 of this thesis. In the present PhD project, an investigation into the relative effects of crystal matrix extract and osteopontin was undertaken as one of several objectives to gain insights into the mechanisms by which the South African black population attains protection against kidney stone formation. The objectives themselves are described in Section 1.9.

1.5 URINARY RISK FACTORS

Urinary risk factors (also known as biochemical risk factors) are regarded as being the main determinants of CaOx crystallization (Robertson et al. 1978; Finlayson et al. 1984; Hess et al. 1996). They are measured in 24 hour urine samples and are calcium, citrate, magnesium, oxalate, uric acid, phosphate, pH and volume. Each of these is discussed below.

1.5.1 Urinary calcium

An abnormally high urinary calcium excretion (hypercalciuria) is one of the most important factors that contribute to a high supersaturation of both CaOx and CaP. Most researchers have reported a normal upper limit of 7.5 mmol per 24 hours for calcium for men and 6.25

mmol per 24 hours for women or 0.10 mmol/kg body weight (for both men and women) (Breslau 1994; Jaeger 1998). Once the 24 hour calcium excretion reaches 10 mmol, there is an increase in the risk of stone formation (Robertson et al. 1978).

Pak (1981) and Buck (1990) reported three different types of hypercalciuria in stone formers namely absorptive (idiopathic), renal and resorptive. Idiopathic hypercalciuria is the most common form and is caused by an overproduction of 1,25-dihydroxy-vitamin D_3 [$1,25(OH)_2D_3$] which controls the transportation of calcium ions through the intestinal cell. Patients with idiopathic hypercalciuria absorb more calcium from food than healthy individuals and this leads to high serum levels of calcium which in turn leads to high urinary calcium (Buck 1990; Breslau 1994; Jaeger 1998; Parmar 2004; Coe et al. 2005). Renal hypercalciuria occurs as a consequence of impaired renal tubular absorption of calcium (Parmar 2004; Coe et al. 2005). This occurs in about 2 % of patients with recurrent stone formation. In resorptive hypercalciuria, increased bone resorption occurs as a result of primary hyperparathyroidism (excessive secretion of parathyroid hormone, PTH) (Pak et al. 1974; Stewart et al. 1982; Mollerup et al. 2002). The major action of PTH is to mobilize calcium from bone, conserve calcium in the kidney and indirectly increase gastrointestinal calcium absorption. Increased PTH levels induce hypercalciuria. This is observed in about 5 % of patients with recurrent stone formation (Mollerup et al. 2002; Parmar 2004).

Dietary calcium is not the only determinant of urinary calcium excretion. Numerous studies have reported that a high sodium intake is associated with increased urinary calcium excretion (Shortt and Flynn 1990; Massey and Whiting, 1996). This is due to an intimate association between renal tubular mechanisms involved in the reabsorption of calcium and sodium ions (Walser 1961; King et al. 1964; Kleeman et al. 1964; Modlin M 1966; Massry et al. 1968; Willis et al. 1969, Matkovic et al. 1995).

1.5.2 Urinary citrate

The association of citrate with calcium stone disease had been reported since 1934 when it was discovered that citrate and calcium form a soluble complex of calcium citrate. By complexing with calcium, citrate lowers the calcium activity which in turn lowers the ion-activity of both CaOx and CaP. Numerous studies have reported urinary citrate to be an inhibitor of CaOx and CaP crystal nucleation, growth and aggregation (Meyer and Smith 1975; Sur and Pandey 1981; Hallson et al. 1983; Kok et al. 1986; Nicar et al. 1987; Pak 1987;

Tiselius et al. 1990; Bek-Jensen and Tiselius 1991; Schwille et al. 1999). Citrate is also known to bind to the CaOx crystal surface, and it is this property that probably explains its influence on both crystal growth and aggregation (Ryall 1997). This gives citrate the potential not to be just a calcium reducing agent, but also a crystal chelator.

The 24 hour urinary excretion of citrate is between 0.6 and 3.5 mmol (Pak 1994; Hesse et al. 1997; Tiselius 1997). Hypocitraturia (low urinary citrate excretion) has been reported to occur in calcium stone formers (Pak et al. 1978; Nicar et al. 1987). Administration of alkaline citrate is therefore recommended for stone formers as it increases urinary citrate thereby reducing the risk of forming stones. This aspect is discussed in detail in Section 1.6.6.

1.5.3 Urinary magnesium

The role of magnesium in CaOx urolithiasis was first suggested in 1932 when it was shown that renal calcification occurred in rats that were fed a magnesium deficient diet (Cramer 1932). This finding was confirmed by Rushton and Spector (1982). In this study, rats were fed magnesium deficient food and intratubular calcium oxalate monohydrate deposits were observed within 24 hours after the administration. The control rats (on regular diet) did not display any renal tubular deposition (Rushton and Spector 1982). Urinary magnesium has been shown to decrease the incidence and recurrence of CaOx stone formation (Trinchieri et al. 1992). This is mainly due to its ability to form soluble complexes with oxalate thereby reducing the free oxalate in urine that could form an insoluble CaOx complex with calcium. It is this capacity of magnesium to chelate oxalate that makes it an inhibitor of CaOx crystallization. Urinary magnesium is also reported to shift the relationship between supersaturation and inhibitory activity towards a situation less favourable for crystal nucleation (Li et al. 1985), aggregation (Domerus et al. 1978; Li et al. 1985) and growth (Ryall et al. 1981).

There have been contradicting reports on urinary magnesium concentration differences between stone formers and healthy individuals. Several studies have reported that no major differences exist between the groups (Robertson et al. 1968; Welshman et al. 1975; Robertson et al. 1978) while others have revealed lower levels in stone formers than controls (Trinchieri et al. 1991, 1992).

1.5.4 Urinary oxalate

Oxalate complexes with calcium in urine to form an insoluble salt of CaOx which is the most common stone-forming compound. Excessive oxalate in the urine (hyperoxaluria) is responsible for about 30 % of calcium stones (Bihl and Meyers 2001; Mente et al. 2006). The upper limit of 24 hour urinary oxalate excretion ranges from 0.4 to 0.5 mmol. Several studies have reported that patients with CaOx stones have a higher urinary oxalate excretion than normal subjects (Robertson et al. 1978; Schwille et al. 1989; Wilson et al. 1989). However, other studies have not detected any differences (Osther et al. 1993; Tiselius 1996; Holmes et al. 1998).

Hyperoxaluria may be due to various factors which include deficiencies in vitamin B6 (Streefland and Donckerwolcke 1989; Curhan et al. 1999) and *Oxalobacter formigenes* (Allison et al. 1986; Sidhu et al. 1999) as well as short bowel syndrome (Khursheed 2002; Kato et al. 2003; Parrish 2005). Severe vitamin B6 deficiencies (usually due to genetic disorders) can result in overproduction of oxalate (Streefland and Donckerwolcke 1989; Parrish 2005). Administration of Vitamin B6 reduces the production of oxalate from glycolate by enhancing the activity of alanine-glyoxylate transaminase, AGT (Danpure and Jennings 1986; Edwards and Rose 1991; Tommasso et al. 2002). Alanine-glyoxylate transaminase inhibits this oxidation process and its activity depends on vitamin B6 (Danpure and Jennings 1986; Nishijima et al. 2003; Danpure 2003, 2005). When AGT is deficient, glycolate is oxidized to oxalate thus increasing the urinary oxalate and risk of CaOx crystallization (Danpure et al. 2003, 2005). *Oxalobacter formigenes* is an oxalate degrading bacterium and low levels in the intestine increase the risk for oxalate absorption and stone formation (Allison et al. 1986; Sidhu et al. 1999). Short bowel syndrome is marked by malabsorption which is the inability of the intestines to absorb fat and nutrients properly (Earnest et al. 1974). In cases of malabsorption, calcium may bind to unabsorbed fat instead of oxalates. This results in excess oxalate being absorbed by the intestines and eliminated through the kidneys (Dobbins and Binder 1976).

Urinary oxalate is the most powerful determinant of the ion activity product of CaOx. Very small increments of oxalate lead to pronounced changes in supersaturation and crystallization (Finlayson 1978; Robertson et al. 1981; Tiselius 2000). It is therefore advisable for kidney stone-formers to reduce their oxalate intake.

1.5.5 Urinary uric acid

Hyperuricosuria plays a major role in uric acid stone formation and may be related to three factors such as low urinary volume, urinary pH less than 5.5 and over-production of uric acid (Coe 1983). Uric acid is the end product of purine (animal protein) metabolism which can be derived from dietary sources such as meat and fish or endogenous production during cell turnover. Therefore dietary intake of foods rich in purine should be avoided to prevent recurrence of uric acid stones (Coe 1978). Hyperuricosuria may be of pathological importance in calcium containing oxalate stones, because uric acid crystals tend to act as a crystal nucleus, around which calcium oxalate crystals precipitate and grow. Indeed, it occurs in 20 % of calcium oxalate stone formers resulting in uricosuric calcium oxalate syndrome (Coe and Kavalach 1974; Coe 1978, 1983).

Allopurinol therapy has been reported to be very effective in reducing uric acid and CaOx stone formation since it blocks the uric acid production and purine absorption (Pak et al. 1978; Ettinger 1991).

1.5.6 Urinary phosphate

Urinary phosphate is directly dependent on the type of diet ingested and is subject to huge variations. It is of great importance in establishing the degree of CaP crystallization (Kok et al. 1988). A normal urinary excretion of phosphate is 35 mmol/day (Hesse et al. 1997). With elevated urinary phosphate concentrations, there is an increased risk of CaP crystallization especially if hypercalciuria is present. However, it is of great importance in inhibition of CaOx crystallization (Schwille 1989; Baumann et al. 2001) for it binds to calcium resulting in reduced complexation of calcium and oxalate.

1.5.7 Urinary pyrophosphate

Urinary pyrophosphate has been reported to inhibit CaOx crystal nucleation and aggregation (Robertson et al. 1973; Meyer and Smith 1975; Ryall 1997). This inhibitory effect is attributed to its potential to irreversibly bind to CaOx crystal surfaces (Ryall 1997). The other main inhibitory feature of urinary pyrophosphate is its capacity to retard and prevent the growth of COM while promoting that of COD crystals (Wikström et al. 1983). As stated earlier, this is of great pathological importance since COD crystals are less adherent to renal

epithelial cells than COM crystals. Pyrophosphate excretion has been reported to be significantly reduced in stone formers compared to healthy controls (Fleish and Bisaz 1962; Wikström et al. 1983).

1.5.8 Urinary pH

Urinary pH is one of the most important determinant factors of the ion-activity product of CaP (Tiselius 2003). It also has an effect on CaOx crystallization. Two individual studies by Kohri et al. (1993) and Pak (1994) have reported that a high urinary pH reduces the risk of CaOx crystallization. An explanation to this is that at a higher pH, more phosphate and citrate ions are dissociated which in turn increases the complexation of calcium and citrate ions thereby reducing the urinary saturation of CaOx (Pak 1994; Hesse et al. 1997). However, the risk of CaP crystallization is concomitantly increased, especially if hypercalciuria is present (Tiselius and Larsson 1992). This was also demonstrated in a study by Højgaard et al. (1999) in which CaP crystals were precipitated at a pH of 6.45 and no CaOx crystals were formed.

A separate study by Hess (Hess 2006) demonstrated that low urinary pH values may be associated with abundant uric acid crystallization and that subsequent stone formation involving this substance can occur. As reported by Hess, the majority of pure uric acid stone formers exhibit a low urinary pH as the main cause of stone formation. Prior to this report, two separate studies by Pak et al. (2002) and Tiselius (2003) involving uric acid stone patients and healthy controls demonstrated that the former had significantly lower values of urinary pH. It is therefore advisable to maintain the urinary pH between 6 and 7 because this range exhibits high inhibition of CaOx crystallization and uric acid stone formation.

A recent study by Rodgers et al. (2005) has shown that the formation of a calcium-citrate-phosphate is favoured as the pH increases. This reduces the amount of free unbound calcium which would otherwise be available to combine with oxalate. The study therefore supports the finding that an increase in pH reduces the urinary saturation of CaOx (Pak 1994; Hesse et al. 1997).

1.5.9 Urinary volume

Urinary volume is yet another extremely important determinant of stone formation. A high volume of urine helps to reduce the relative supersaturation of the crystal components. In

addition to this, high urinary volume indicates high urine flow rate which in turn tends to wash out any crystals that have formed, thereby reducing the risk of crystal-cell adhesion (Borghi et al. 1996). High fluid intake (usually water) is therefore routinely recommended for kidney stone patients (Strauss et al. 1982; Pak et al. 1980; Curhan et al. 1998). Some studies encourage urinary outputs in excess of three liters per day (Sakhaee et al. 1987; Menon and Resnick 1999).

The significance of increasing urinary volume on kidney stone reduction was demonstrated in a randomized clinical trial conducted by Borghi et al. (2002). In this study, stone recurrence occurred in only 12 % of the 99 patients who maintained a urinary volume of about 2.6 L/day over five years. However, stones recurred in 27 % of the 100 patients in the control group, whose urinary volume was about 1.2 L/day.

Apart from water, other fluids can be safely consumed and are associated with a positive effect. A study conducted by Curhan et al. (1998) demonstrated that caffeinated and decaffeinated coffee, tea and wine were significantly associated with a lower risk of kidney stone formation whereas grapefruit was associated with a higher risk. This study was supported by other researchers who showed an inverse association of coffee and tea with the risk of stone formation (Shuster et al. 1985; Krieg 2005).

1.6 DIET AND SUPPLEMENTS

Diet can significantly affect urinary composition. As such, certain foods and supplements may be lithogenic whereas others may be protective. Some of the most important of these in both categories are dietary calcium, salt and phytate and supplemental citrate and bicarbonate. These will be discussed in detail in the following paragraphs.

1.6.1 Dietary Calcium

Dietary intake and urinary calcium excretion are the most important determinants of calcium retention in the body (Nordin and Marshall 1998). Low calcium intake and high losses of calcium in urine could reduce calcium distribution into bone (Matkovic et al. 1995). In the past there used to be contradicting reports on the effect of dietary calcium on CaOx urolithiasis. Some researchers believed that high dietary intake of calcium increases the risk of forming kidney stones (Robertson et al. 1981; Breslau 1994). Robertson (1999) also

reported that one of the most common and effective recommendations given to calcium stone formers is to reduce the intake of calcium.

Contrary to these reports, several studies showed that a reduction in calcium intake not only increases the risk of calcium stone formation, but also causes a negative calcium balance and further loss of bone tissue (Coe et al. 1992; Curhan et al. 1993; Curhan 1999; Lemann 2002). In their two separate studies, Curhan and his team (Curhan et al. 1993, 1997) revealed a 34 % lower incidence of kidney stone formation in subjects with a high intake of calcium compared to others with a low intake. These studies are in agreement with a recent study by Heller et al. (2003) which reported that a high calcium diet conferred an alkali load that led to an increase in urinary pH and citrate. Besides having a beneficial effect on CaOx saturation, it also significantly decreased the urinary saturation of CaP in this study. Curhan et al. (2004) also discovered that in previously non-stone forming younger women, higher intake of dietary calcium was inversely related to the risk of stone formation.

Low dietary calcium has long been recognized as an enhancer of oxalate absorption in the gut and subsequent excretion in the urine (Epstein 1968; Marshall et al. 1972). Recent studies have confirmed this (Menon and Resnick 1999; Lemann 2002). Therefore, a decrease in calcium intake without a concomitant decrease in oxalate intake leads to increased urinary oxalate which subsequently leads to a high risk of stone formation (Hughes and Norman 1992; Liebman and Chai 1997). In addition, dietary restriction of calcium in stone formers increases their risk of bone loss whereas adequate calcium intake is required for the constant process of bone resorption and formation (Shortt and Flynn 1990; Nordin et al. 1991).

In the light of these reports, it is therefore recommended that calcium stone patients consume dietary calcium within the Recommended Daily Allowance of 1,000 to 1,200 milligrams daily with a well-maintained dietary intake of sodium and animal protein.

1.6.2 Dietary Protein

An increase in protein consumption has been reported to cause an increase in urinary calcium excretion (Robertson et al. 1980; Kerstetter et al. 2003). Robertson (1999) demonstrated a significant increase of 23 % in urinary calcium after an increase in animal protein intake to 34 g/day. This study also reported an increase in urinary oxalate. This finding was also supported by a recent study by Massey (2005). It was reported that a 10 gram increase in

dietary protein increases urinary calcium by 16 milligrams, and doubling dietary protein leads to a 50 % increase in urinary calcium excretion (Massey 2005). This direct relationship between dietary protein and urinary calcium excretion is attributable to the proposition that dietary protein increases the endogenous acid production which subsequently leads to metabolic acidosis (Brokis et al. 1982; Bailey JL 1998; Boirie et al. 2000). Metabolic acidosis results in bone calcium resorption and increased calcium excretion (Brokis et al. 1982). This suggests that high protein intake not only has a negative effect on CaOx urolithiasis, but also osteoporosis as well.

As mentioned earlier, a high purine-rich animal protein intake results in high uric acid, high pH and low citrate excretion which in turn leads to a high risk of uric acid and CaOx stone formation (Coe 1978; Trinchieri et al. 1991; Curhan et al. 1993; Nguyen et al. 2001). A recent study by Lemann (2002) reported that vegetarians form stones at one-third the rate of those eating animal protein. This is attributable to high urinary uric acid excretion following animal protein intake (Breslau et al. 1994). Conversely, vegetarian diets result in reductions in urinary uric acid (Breslau et al. 1994). A study conducted on 18 hypercalciuric stone formers found that a 15-day protein restriction had positive effects on urinary stone formation risk factors (Giannini et al. 1999; Krieg 2005). In this study, significant decreases in urinary calcium, uric acid, phosphate, and oxalate and a beneficial increase in urinary citrate were observed.

1.6.3 Dietary phytate

Several studies have reported that phytate is one of the most powerful inhibitors of CaP and CaOx crystallization (Graf and Eaton 1990) and thus prevents urolithiasis (Grases and March 1989; Grases et al. 1994 (a) and (b), 1998, 2000 (a) and (b)). Normal human urine has been reported to contain approximately 2.5 mg/L of phytate, but it is significantly lower in the urine of stone formers (1.5 mg/L) (March et al. 1998; Grases et al. 1999). There is an important positive correlation between dietary intake and urinary excretion of phytate (Taylor and Curhan 2004).

A clinical study conducted by Conte et al. (1989) on CaOx stone formers showed that even low consumption doses of phytate significantly reduced the risk of developing stones. The potency of phytate in reducing risk of stone formation rests in its calcium binding affinity (March et al. 1998; Grases et al. 2000 (a)). It has been shown that even at low doses

of phytate, 1- 5 % of the extra ingested phytate is excreted in urine (Grases et al. 2000 (b)). The relationship between CaOx urolithiasis and phytate ingestion was supported by a comparative study by Grases et al. (1999) between healthy subjects and stone formers, who showed that the consumption of fibre which is rich in phytate is lower in stone formers. Modlin (1980) also reported that the dietary intake of phytate in the white South African population is lower than in their black counterparts.

1.6.4 Dietary carbohydrates

Several studies have reported that high carbohydrate intake results in increased urinary calcium excretion (Raskin et al. 1978; Wood and Allen 1983; Iguchi et al. 1993; Coe and Parks 1994; Taylor and Curhan 2004). Carbohydrates also stimulate endogenous synthesis of oxalate which is eventually excreted in urine (Lekcharoensuk et al. 2001). With high urinary concentrations of calcium and oxalate, the risk of forming stone is more pronounced as these are the main urinary CaOx risk factors. A diet low in carbohydrates is therefore recommended for stone-forming individuals.

1.6.5 Dietary Sodium Chloride

The effect of dietary sodium in CaOx stone formation has been extensively studied by many researchers since the 1930s (Aub et al. 1937; Kleeman et al. 1964; McCarron et al. 1981; Breslau et al. 1982; Silver et al. 1983; Burtis et al. 1994). Dietary sodium is a well-known determinant of urinary calcium excretion (Burtis et al. 1994) and reducing dietary salt (sodium) helps to reduce the amount of calcium in the urine, which in turn reduces the tendency for calcium stone formation (King et al. 1964; Muldowney et al. 1982; Shortt and Flynn 1990; Shortt et al. 1998). It has been reported that each 100 mmol increase in daily sodium intake is associated with an approximate increase of 1 mmol in urinary calcium excretion (Nordin et al. 1993; Lemann et al. 1999). As mentioned earlier, this positive correlation between sodium intake and urinary calcium excretion is due to an intimate association between renal tubular mechanisms involved in the reabsorption of these two ions (Matkovic et al. 1995).

This finding has been supported by a recent study which investigated the effect of dietary salt intake on bone turnover markers and CaOx kidney stone risk (Massey 2005). This study showed that a sodium chloride intake of 200 mmol led to an unfavourable increase in

urinary calcium and urinary oxalate. However, no changes in urinary magnesium and citrate were observed.

According to a hypothesis developed by numerous researchers, the salt induced increase in urinary calcium causes a huge drop in plasma ionized calcium, which in turn stimulates parathyroid hormone (PTH) release (Goulding 1980; Goulding et al. 1986; Shortt and Flynn 1990; Evans et al. 1997). Increased PTH stimulates bone resorption which in turn releases calcium into the plasma and returns serum calcium to its physiologic level.

An increase in dietary salt intake not only has an unfavourable effect on the risk of kidney stone formation but also influences bone loss which eventually leads to osteoporosis (Goulding and McParland 1990; Shortt and Flynn 1990; Massey and Whiting 1996; Ginty et al. 1998; Lin et al. 2003; Massey 2005). Several studies had been undertaken to determine the effect of sodium chloride ingestion on bone formation and resorption turnover markers mainly in pre- and post-menopausal women (Goulding 1981; Goulding and Lim 1983; Shortt and Flynn 1990; Matkovic et al. 1995; Evans et al. 1997; Lietz et al. 1997; Ginty et al. 1998; Sellmeyer et al. 2002; Lin et al. 2003; Massey 2005; Wigertz et al. 2005). These studies report contradicting results as some of them have found no change (Ginty et al. 1998; Evans et al. 1997; Lietz et al. 1997; Lin et al. 2003) while others have reported an increase in bone turnover markers (Evans et al. 1997; Wigertz et al. 2005). Not surprisingly, decreases have also been reported (Evans et al. 1997; Sellmeyer et al. 2002; Massey 2005).

Of great interest is a study comparing in calcium retention in response to dietary salt in adolescent girls of different races (Wigertz et al. 2005). In this study black adolescent girls showed a higher retention of calcium than their white counterparts who had the same calcium intake. Prior to this report, studies on black and white adolescent girls had also demonstrated a higher calcium balance of 12 % in the bone mass of black relative to white women (Bryant et al. 2003) which was consistent with a 10 - 13 % higher adult bone density reported in the former group (Kleerekoper et al. 1994; Hui et al. 2003; Palacios et al. 2004). From these studies it was concluded that notwithstanding the importance of sodium as a determinant of urinary calcium retention, race could be a major determinant as well.

These studies are extremely interesting in the context of the South African phenomenon in which kidney stone formation is almost non-existent in the black population. Indeed, the relative renal handling of sodium chloride in South Africa's two population

groups poses an intriguing question. As such, this aspect was adopted as another objective for the present PhD thesis.

1.6.6 Supplemental Citrate

The significance of alkaline citrate therapy in preventing calcium stone recurrence has been reported in many clinical studies (Lemann et al. 1989; Pak 1987, 1991, 1994; Schwille 1985, 1987, 1997; Rumenapf and Schwille 1987; Whalley et al. 1998).

Alkali therapy has been recognized as a powerful agent for treating nephrolithiasis since Sir Astley Cooper in 1826 prescribed liquor potasse, potassium carbonate and sodium carbonate for uric acid stones (Atsmon et al. 1963). Since then, stone patients have been treated with alkali agents either in the form of bicarbonate or citrate salts (Howard 1954; Gregory et al., 1981; Sakhaee et al. 1991; Pak 1991, 1994). Supplemental citrate is the most common therapeutic agent that is used to prevent CaOx and uric acid stones. The powerful inhibitory potency of citrate rests in its ability to alkalinize urine, and to form soluble complexes with calcium. Such complexation reduces urinary supersaturation for CaOx and CaP and retards the nucleation, aggregation and growth as well as agglomeration of preformed CaOx crystals (Pak and Fuller 1983; Kok et al. 1986; Fan et al. 1995; Parks et al. 1996; Laube et al. 2002 (a); Ettinger et al. 1997; Byer and Khan 2005).

The therapeutic effect of various different citrate salts as alkali therapy on CaOx crystallization inhibition has been tested extensively. These salts include potassium citrate (Sakhaee et al. 1983; Pak et al. 1985; Hofbauer et al. 1994; Whalley et al. 1998; Sellmeyer et al. 2002), sodium citrate (Sakhaee et al. 1983; Allie-Hamdulay and Rodgers 2005), magnesium citrate (Schwille et al. 1999), potassium-magnesium citrate (Ettinger et al. 1997), calcium citrate (Sakhaee et al. 2005), calcium-sodium citrate (Schwille et al. 1997) and sodium-potassium citrate (Pak and Adams 1987; Achilles et al. 1990; Hofbauer et al. 1994). Numerous investigators have reported reductions in the rate of recurrent stone formation by 60 % to 96 % following administration of alkali citrate therapy (Pak et al. 1985; Preminger et al. 1985 (a) and (b); Pak and Fuller 1983; Pak and Peterson 1986; Ettinger et al. 1997). 56 % of stone forming patients who experienced severe pain, reported spontaneous elimination to be painless and stone formation was decreased by 31 % following citrate therapy (Hofbauer et al. 1994; Pak and Peterson 1986). These data demonstrate that alkali citrate therapy for CaOx stone formers might be justified on the basis of virtually no incidence of side effects

and physiological processes remain unaffected (Hofbauer et al. 1994; Pak and Peterson 1986).

A comparative study (on CaOx stone formers) on the effect of potassium citrate and sodium citrate showed that both salts produced a significant increase in urinary citrate excretion and in pH (Sakhaee et al. 1983). However, while potassium citrate significantly reduced urinary calcium excretion, the same effect was not achieved by sodium citrate. Despite this, the therapeutic potency of sodium citrate in the management of CaOx urolithiasis risk factors in male and female controls and stone formers was recently demonstrated in the Kidney Stone Research Laboratory at the University of Cape Town (Allie-Hamdulay and Rodgers 2005). This study reported an increase in citrate excretion and urinary pH and a concomitant decrease in calcium excretion in all groups following administration of sodium citrate. Crystallization experiments conducted in this study support the view that sodium citrate could be a potential prophylaxis in the management of CaOx urolithiasis (Allie-Hamdulay and Rodgers 2005).

The present PhD project proposes to extend the aforementioned study by Allie-Hamdulay and Rodgers to involve an in-depth investigation into the renal handling of sodium citrate by the black and white population groups, with a view to exploring whether differences exist in the renal handling thereof by the respective race groups, and if so, whether these differences might contribute towards understanding why stone rarity occurs in the former group.

1.6.7 Supplemental Bicarbonate

As mentioned earlier, alkali therapy has long been recognized as a powerful agent for treating nephrolithiasis (Atsmon et al. 1963). However, the therapeutic potential of alkali therapy depends on attaining a metabolically optimal range of plasma bicarbonate that is higher than that comprising its normal range. To accomplish this, one needs to understand the effect of alterations in acid–base balance on urinary calcium excretion and calcium balance. Metabolic acidosis and increased acid production are accompanied by increased urinary calcium excretion which arises as a consequence of inhibition of net renal tubular calcium reabsorption (Lemann et al. 1965, 1966, 1967, 1986; Morris and Sebastian, 2002). This acidosis also has a negative impact on osteoporosis as it decreases intestinal calcium reabsorption and stimulates bone resorption, thereby affecting calcium balance (Lemann et al.

1966, 1979). Metabolic acidosis can be reversed by alkali therapy which leads to metabolic alkalosis (McSherry and Morris 1978; Osther et al. 1993; Morris and Sebastian 2002).

Metabolic alkalosis is achieved by administration of bicarbonate which is accompanied by reduction in urinary calcium excretion (Lemann et al. 1979). Bicarbonate has been investigated in the form of potassium (Sakhaee et al. 1983; Lemann et al. 1989; Sebastian et al. 1994, 2005; Frassetto et al. 2005) and sodium bicarbonate (Sakhaee et al. 1983; Lutz 1984; Lemann et al. 1989). Both supplemental forms have been reported to reduce urinary calcium excretion and increase positive calcium balance, plasma bicarbonate and a reverse induction in net renal acid excretion (Morris and Sebastian 2002). Bicarbonate not only reduces the risk of stone formation, but also osteoporosis. Several researchers have reported an improved external balance of calcium as demonstrated by reductions in the urinary excretion rate, followed by an increase in serum concentrations of bone formation markers of osteocalcin and a decrease in urinary concentrations of bone resorption markers (Lutz 1984; Lemann et al. 1989; Sebastian et al. 1994, 2005; Frassetto et al. 2005; Sakhaee et al. 2005).

As was the case with sodium chloride and sodium citrate (sections 1.6.5 and 1.6.6), an investigation into the relative renal handling of sodium bicarbonate in South Africa's two population groups is warranted. Hence, such a study is yet another objective of the present PhD project.

1.7 KIDNEY STONES AND OSTEOPOROSIS

Osteoporosis is a chronic, slowly developing disease that is characterized by a reduction of bone mass and impairment of the structural integrity of the bone, making patients more susceptible to bone fractures (Clowes and Eastell 2000). It generally occurs with increasing age and in post-menopausal women. The pathogenesis of osteoporosis, fragility fractures and metabolic disease is associated with many factors such as genetic, environmental, biomechanical, chronic disease and the effects of endogenous hormones. Throughout life, bone is in a dynamic state of continuous resorption and formation and this process is called bone remodelling. In childhood and early adulthood, formation exceeds resorption so that bone density increases and plateaus in the age range of 30 to 40 years. After 40 years of age, resorption exceeds formation, and bone density decreases throughout the rest of life. Bone

remodeling, or turnover, consists of two opposing activities: the breakdown (resorption) of old bone by osteoclasts, and the formation of new bone by osteoblasts (Delmas 1990, 1993). Osteoporosis is characterized by an increase in bone resorption and a decrease in bone formation (Price et al. 1983; Delmas 1993, 2000).

Bone turnover can be assessed by measuring levels of various biochemical markers which are released into the blood or urine (Delmas 1992, 2000). Markers exist for both bone formation and bone resorption. Bone formation markers are released during osteoblast synthesis of new bone protein matrix and can be only measured in blood. Bone resorption markers are released into the circulatory system as by-products of osteoclast action on bone. Resorption markers can be measured in both blood and urine. Some of resorption markers result from the breakdown of type I collagen, which is a major component of bone matrix, comprising about 90 % of the bone's organic content. Examples of both categories of bone markers are given in Table 1.1 and some are discussed below.

Table 1.1: Biochemical markers of bone turnover

Bone formation markers	Bone resorption markers
Total alkaline phosphatase (TALP)	Calcium
Bone alkaline phosphatase (BALP)	Hydroxyproline
Procollagen-I extension peptide	Pyridinolines (PYD)
Osteocalcin (OC)	Deoxypyiridinolines (DPD)
	COOH-terminal telopeptides (CTX)
	NH_2-terminal telopeptides (NTX)

Alkaline phosphatase (ALP) and osteocalcin are the most widely used bone formation markers.

ALP is a ubiquitous enzyme that plays an important role in osteoid formation and mineralization. Serum ALP activity is the most commonly used marker of bone formation, but it lacks sensitivity and specificity (Seibel 2005). Nevertheless, studies by Delmas (1992, 1993) and Osther et al. (1993) have shown that its activity increases with aging in adults, especially in women after menopause. In patients with vertebral osteoporosis, values are either normal or slightly elevated and poorly correlated with bone formation (Delmas 1993).

Osteocalcin is predominantly synthesized by the osteoblasts and incorporated into the extracellular matrix of bone, but a fraction of newly synthesized osteocalcin is released into the circulatory system (Price et al. 1983). Circulating osteocalcin has a short elimination half-life and is rapidly cleared by the kidney (Delmas et al. 1983). Serum osteocalcin is a sensitive and specific marker for osteoblastic activity that accurately reflects both gradual age-related and accelerated postmenopausal increases in bone turnover (Johansen et al. 1988). It contains three γ-carboxyglutamic acid (Gla) residues, which are responsible for the calcium binding properties of this protein (Johansen et al. 1988; Delmas 1993). It is also considered as a specific marker of osteoblast function as its levels correlate with bone formation rates.

Pyridinolines (PYD) and deoxypyridinolines (DPD) are the hydroxypyridium crosslinks of collagen and are formed during the extracellular maturation of fibrillar collagens and are released upon the degradation of mature collagens (Robins 1999; von der Mark 1999). Their measurement is not influenced by the degradation of newly synthesized collagens and independent of dietary sources. They are excreted in urine in free form (approximately 40 %) and in peptide-bound form (60 %). Urinary PYD and DPD are increased by 50 - 100 % at the time of menopause and return to premenopausal levels with estrogen therapy (Johansen et al. 1988). In patients with vertebral osteoporosis, the urinary DPD levels are correlated with bone turnover. Both PYD and DPD appear to be more sensitive than hydroxyproline as markers of bone resorption, and are also significantly increased in patients with primary hyperparathyroidism, malignant hypercalcemia and hyperthyroidism (Delmas et al. 2000; Seibel 2005). These two bone resorption markers are relatively specific for bone turnover and they do not appear to be metabolized in vivo prior to their urinary excretion. PYD and DPD are at their highest peak during the night and were lowest during the afternoon and this probably reflects a nocturnal increase of bone turnover and resorption (Eastell et al. 1992; Kraenzlin and Seibel 1999; Seibel 2005).

As stated previously, osteoporosis, just like kidney stones is a multi-factorial disease. It is correlated with age, gender and ethnicity (Delmas et al. 2000). Khosla et al. (1997) and Fatayerji and Eastell (1999) reported that children have significantly higher biochemical markers than adults, particularly at puberty when they have 2-10 times the levels found in adults. In women there is a huge increase in bone turnover markers at menopause, while for men there is no change with age (Khosla et al. 1997; Fataverji and Eastell 1999).

Higher levels of bone markers have been reported in young men in their third and fourth decade than young women, but in older men the levels are lower in men than in postmenoupausal women (Delmas et al. 2000). A comparative study of black and white populations in France has reported that in children and young adults, bone resorption markers are 20 % lower in black subjects than in white subjects (Delmas et al. 2000).

Interestingly, it has been reported that serum osteocalcin and urinary hydroxyproline are significantly decreased and increased, respectively, in kidney some formers with renal tubular acidosis (Osther et al. 1993). It has also been reported that some patients with renal tubular acidosis had either kidney stones or nephrocalcinosis (Weger et al. 1999). Citrate treatment has been reported to inhibit osteoclastic resorption and to stimulate osteoblastic formation (Sebastian et al. 1994; Bushinsky, 1996).

As mentioned in the previous pages, the effect of sodium chloride (Muldowney et al. 1982; Shortt et al. 1998; Shortt and Flynn 1990; Ginty et al. 1998; Sellmeyer et al. 2002; Lin et al. 2003; Massey 2005; Wigertz et al. 2005), bicarbonate (Lemann et al. 1965, 1966, 1967, 1986; Morris and Sebastian et al. 2002; Frassetto et al. 2005; Sakhaee et al. 2005) and citrate (Sebastian et al. 1994; Bushinsky 1996; Ettinger et al. 1997; Laube et al. 2002 (a); Sellmeyer et al. 2002) on kidney stone formation as well as bone turnover markers of osteoporosis has been extensively studied, albeit that the latter investigations have been mainly in pre- and post-menopausal women. Thus, a further objective of the present PhD project is to investigate the effect of these salts on bone turnover markers in the South African racial context.

1.8 UROLITHIASIS IN SOUTH AFRICA

As stated previously, the incidence of kidney stones in the black South African population is extremely rare (< 1 %) compared to 12 - 15 % reported in the white population (Modlin, 1967; Meyers et al. 1994, Whalley et al. 1998; Pinnock et al. 2004). This prevalence rate in the white population is similar to that in Westernized countries (Modlin 1967; Whalley et al. 1998). The rarity of stones in the black South African population group is most likely due to a multitude of factors which may or may not include differences between their urine chemistry, urinary proteins and dietary habits (Modlin 1967, 1980; Beukes et al. 1987; Pinnock et al. 2004).

1.8.1 Urine Chemistry

In an attempt to understand and explain the low stone incidence in the black South African population, the Kidney Stone Research Laboratory (KSRL) at the University of Cape Town has extensively investigated the aforementioned factors. Studies by this group and others have revealed that black subjects have lower urinary calcium (Modlin 1967; Whalley et al. 1998; Rodgers and Lewandowski 2002), lower urinary citrate, lower urinary phosphate and lower urinary pH (Modlin 1967; Lewandowski et al. 2001) and higher urinary oxalate (Rodgers and Lewandowski 2002). Several of these results are surprising as they are counter-intuitive. For example, the lower urinary citrate and pH and the higher urinary oxalate would be expected to *increase* the risk of CaOx stone formation. On the other hand, while the lower urinary calcium and phosphate might culminate in a lower stone incidence rate in black subjects, they cannot account for the virtual absence of stones in this group as their levels lie within the normal range.

1.8.2 Biochemical Factors: Urinary Proteins

Since routine urine composition cannot explain the difference in kidney stone occurrence in South Africa's two population groups, the Kidney Stone Research Laboratory at the University of Cape Town has extensively investigated the role of several urinary proteins in this regard. Studies on the so-called crystal matrix extract (CME) derived from precipitated calcium oxalate monohydrate (COM) crystals showed that the extract was an inhibitor of CaOx crystal nucleation and aggregation, and that the extract from black subjects was superior in this regard to that from whites (Durrbaum et al. 2001).

Studies on UPTF1 (isolated from COM-CME) showed that it is a potential inhibitor of CaOx crystal growth and aggregation and that the protein isolated from the urine of black subjects was superior to that from white subjects (Durrbaum et al. 2001; Webber et al. 2002). Similarly for THP, the form isolated from the urine of black subjects was found to be a stronger inhibitor of CaOx crystal aggregation than that from white subjects (Craig et al. 1999, 2000, 2001). Albumin isolated from black subjects has also been shown to be a superior inhibitor of CaOx crystal growth and aggregation than that from white subjects (Rodgers et al. 2006). Finally, bikunin isolated from the two race groups demonstrated strong inhibition of CaOx crystal growth and aggregation and a weak inhibition of CaOx crystal

nucleation; however aggregation inhibition by bikunin from black subjects was superior (Mabizela PhD Thesis, 2006).

An interesting result which emerged from all of these studies was the observation that a synergistic relationship exists between the inhibitory performance of the protein and the chemical composition of the urine milieu in which it was tested. Thus, UPTF1 isolated from black subjects was found to be a more efficient inhibitor of CaOx crystal growth and aggregation than that isolated from white subjects in their endogenous urines (Durrbaum et al. 2001; Webber et al. 2002). THP from black subjects inhibited CaOx crystal growth in urines from both black and white subjects, whereas THP from the white group promoted CaOx crystal growth in its own urine but inhibited this mechanism in urine from black subjects (Craig et al. 1999, 2000, 2001). On the other hand, while albumin from both groups inhibited CaOx crystal growth and aggregation in their endogenous urines, that isolated from black subjects was more efficacious (Rodgers et al. 2006). Bikunin inhibited CaOx crystal growth and aggregation in both urines and that isolated from black subjects was superior in this regard (Mabizela PhD thesis, 2006).

1.8.3 Diet and Supplements

As in urine composition studies, few adult population studies have been undertaken to identify dietary similarities and differences in South Africa's black and white population groups (Segal 2002; Steyn 2003).

Oxalate

The South African black population has been reported to have a high dietary intake of oxalate (because of the regular intake of spinach) (Viljoen and Gericke 2001), a low intake of calcium (because of lactose intolerance) (Viljoen and Gericke 2001) and a low magnesium intake (correlated with their low ingestion of vegetables) (Whalley et al. 1998; Charlton et al. 2005). These dietary habits are widely regarded as hyperoxalurogenic i.e. favouring the excretion of relatively high urinary oxalate. However, despite these eating habits, urinary oxalate in this race group lies within the normal range (Whalley et al. 1998; Rodgers and Lewandowski 2002; Lewandowski et al. 2005).

To test the effect of dietary challenges and the ability to handle oxalate in the two South African population groups, three studies were conducted with these two groups (Lewandowski et al. 2001, 2005; Rodgers and Lewandowski 2002). A high oxalate-low calcium diet given to both groups significantly changed the urinary oxalate only in whites but not in blacks (Lewandowski et al. 2001). Two other independent diets, one low in calcium and the other high in oxalate were administered. The low calcium diet resulted in an increase in oxaluria in blacks whereas the urine biochemistry was not changed in whites (Rodgers and Lewandowski 2002). Despite the induced oxaluria in blacks, the relative supersaturation of CaOx decreased significantly (Rodgers and Lewandowski 2002). The high oxalate diet induced a significant increase in urinary pH in whites and an increase in urinary citrate in blacks (Rodgers and Lewandowski 2002).

These results demonstrated that the South African black population handles dietary hyperoxalurogenic challenges in a different way to that of their white compatriots.

Salt

Interestingly, the black South African population has been reported to have a diet that is high in salt relative to the white group (Modlin 1967; Whalley et al. 1998). However, despite this, their urinary calcium is lower (Rodgers and Lewandowski 2002). This suggests once again that the South African black population group has a different handling mechanism of lithogenic agents than their white compatriots.

Even though high sodium intakes may be a risk factor for osteoporosis and CaOx urolithiasis, some sodium salts such as sodium citrate and sodium bicarbonate have been shown to have no significant effect on urinary calcium in whites (Allie–Hamdulay and Rodgers 2005). An obvious question which arises is whether such salts have any influence on urinary calcium in South African blacks. Indeed, this forms the basis of yet another objective in the present PhD project.

1.9 OBJECTIVES OF THIS THESIS

As stated in the opening paragraph of this thesis, the presence of stone-prone and stone-free population groups in South Africa provides basic scientists with a unique opportunity to study kidney stone pathogenesis with a view to gaining insights into the key factors for stone formation on the one hand, and stone prevention on the other. Other sections in Chapter 1 have drawn attention to various intriguing phenomena in this context. These provide the basis for the following objectives which were undertaken for the present thesis:

- To investigate the relative inhibitory capacities towards CaOx crystallization, of urine from healthy black and white male subjects, with reference to urinary macromolecules of size > 10 kD.
- To investigate the relative inhibitory capacities towards CaOx crystallization of crystal matrix extract (CME) derived from calcium oxalate dihydrate crystals precipitated from the urine of healthy black and white male subjects.
- To repeat the CME studies in ultrafiltered urine from both race groups using a cross-over design, with a view to exploring the influence of the respective urine milieus on the inhibitory performance of CME.
- To investigate the inhibitory capacity of commercial osteopontin in ultrafiltered urine from both race groups, with a view to exploring the influence of the respective urine milieus on the performance of the protein.
- To investigate the effects of the oral administration of three different sodium salts (sodium chloride, sodium citrate and sodium bicarbonate) on urinary calcium and other biochemical and physicochemical risk factors for CaOx stone formation in black and white healthy male subjects, with a view to exploring whether the renal handling of these salts is different in the two population groups.
- To investigate the effects of the aforementioned sodium salts on markers of bone turnover, with a view to further exploring whether the renal handling thereof is different in the two population groups.
- To investigate whether urinary alterations following oral administration of the aforementioned sodium salts change the crystallization properties of urine in the two population groups.

CHAPTER TWO

GENERAL METHODS

2.1 INTRODUCTION

This chapter mainly focuses on the general methods that were used for the various studies described in this thesis. These experiments commenced with the collection and treatment of 24 hour urine samples followed by their physicochemical analyses and the determination of CaOx metastable limits (MSLs), CaOx crystallization kinetics involving particle number, volume and volume-size distributions, ^{14}C-oxalate deposition kinetics and sedimentation experiments to assess CaOx crystal nucleation and aggregation inhibition. The studies on aggregation were supplemented with zeta potential measurements. Finally, scanning electron microscopy was used to visualize deposited crystals in an attempt to confirm mechanisms arising from interpretation of the data obtained in the aforementioned experiments.

2.2 METHODS

2.2.1 Urine collection, treatment and physicochemical properties

Age matched black and white healthy males with no history of kidney stone formation participated in the various studies described in this thesis. 24 hour urine samples were collected from each. Each sample was tested for haematuria and nitrite using urinalysis test strips (Medi Test Combi 5N, Macherey-Nagel; Düren), and those that tested positive for either were discarded. All urine samples were analysed for sodium, potassium, calcium, oxalate, uric acid, citrate, chloride, magnesium, phosphate, sulphate and creatinine. Sodium, potassium, magnesium and calcium were measured using atomic absorption spectroscopy (Varian 1275 Model; Australia) (Willis 1969; Trudeau and Freier 1967; Fernandez and Kahn 1971) while commercially available assay kits from Sigma Aldrich and Boehriner Mannheim were used for oxalate (Chiriboga 1963) and citrate, respectively (Gruber and Möllering 1966). Chloride was determined using a chloride sensitive electrode. Creatinine (Rock et al. 1986), phosphate (Dryer and Routh 1963), and urate (Fossati et al. 1980) were measured using Synchron LX assay kits (Beckman Coulter Inc.).

The urinary relative superaturations of CaOx, brushite and uric acid were calculated using the computer programme EQUIL2 (Werness et al. 1985). The Tiselius Risk Index (TRI) was calculated for each sample using urine composition data (Tiselius 1982). The Tiselius Risk Index formula of 1982 was used to be consistent with other urinary studies at the University of Cape Town involving South African black and white subjects. Urine composition data were analysed statistically using analysis of variance (ANOVA) and if $p \leq 0.05$ the results were considered statistically significant. Variances were pooled on the assumption that groups had equal variances.

Albumin in the urine in any more than the most trifle quantity is considered a clear indicator of kidney disease with altered (diseased) renal metabolism affecting renal handling of other urine constituents as well. In the present study, the samples were tested for protein and blotted for albumin (chapter 4) and virtually none was detected.

2.2.2 Crystallization Experiments

Crystallization experiments except for crystal aggregation were conducted in duplicates for each sample and the mean of the two values was reported. The two measurements for each sample were more than 85 % reproducible.

CaOx Metastable Limit (MSL)

Urine samples were prepared for the determination of the CaOx metastable limits by filtering successively through a 0.75 µm pre-filter (Macherey-Nagel; GmBH and Co., Germany) and 0.45 µm nitrocellulose filter (Sartorius AG, Germany). The MSL of each sample was determined following the method described by Ryall and co-workers (Ryall et al. 1985). Aliquots (10 mL) of each sample were added into Coulter cups and incubated at 37 °C in a shaking water bath at 100 rpm (Labcon Marketing Services, Johannesburg). The aliquots were dosed with 100 µl of progressively increasing concentrations of sodium oxalate (Na_2Ox; 0.015 mol/L to 0.195 mol/L) at 2 minute intervals and incubated for a total of 30 minutes each. The particle number and volume were measured using a Coulter Multisizer I (Coulter Electronics Ltd., England) fitted with a 140 µm orifice (2.8 - 90.0 µm particle size range). The concentration of Na_2Ox corresponding to the dose which caused a sudden increase in

particle number was taken as measure of the metastable limit of the particular sample under investigation. A representative curve which shows the determination of the metastable limit is shown in Figure 2.1. The concentration which corresponds to the metastable limit is shown by a red dot.

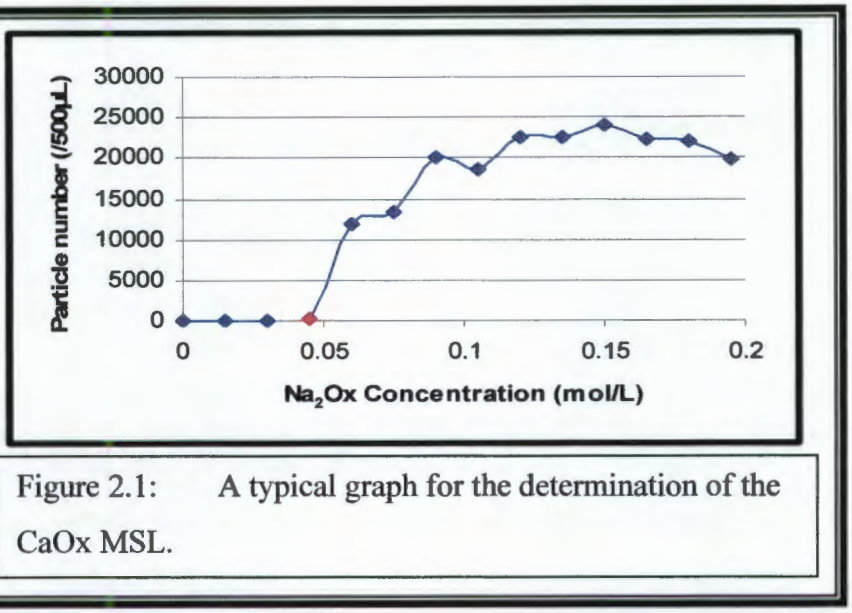


Figure 2.1: A typical graph for the determination of the CaOx MSL.

CaOx Crystallization Kinetics

Once the MSL of each sample had been determined, 100 mL aliquots were incubated for 10 minutes at 100 rpm and at 37 °C in a shaking water bath (Labcon Marketing Services, Johannesburg). An aliquot of 10 % (v/v) Na_2Ox equivalent to 30 mmol/L above the previously described MSL was then added to the sample to induce crystallization. Thereafter, the sample was incubated and a zero time measurement of particle number, volume and size was recorded using the Coulter Counter. Incubation continued for 2 hours during which time Coulter measurements were made at 30 minute intervals.

Scanning Electron Microscopy (SEM)

All urine samples were dosed with aqueous Na_2Ox in the kinetics experiment as described in the previous paragraph prior to examination by scanning electron microscopy. This procedure was adopted because natural undosed samples in all experiments did not reveal crystals large enough to be visualized by SEM. Despite this exogenous method for producing crystalluria, comparison of different samples in this way is regarded as being valid and appropriate, as all urines were treated in the same way.

After the 2-hour incubation period, the samples were filtered through 0.22 μm filters and dried at room temperature for 1 hour. Once the filter papers were dry, the crystals were mounted on aluminium stubs and were splutter-coated for 10 minutes with 3 – 5 nm of Au/Pd (Bio-Rad, SEM Coating System). The deposited crystals were then viewed using a Leica S440 scanning electron microscope (Leica Cambridge Ltd, Cambridge, England) operating at a working distance of 10-15 mm, an accelerating voltage of 10 kV and a probe current of 20-30 pA. Micrographs for a particular series of experiments were always recorded at the same magnification so that valid comparisons could be made. Obviously, the actual magnification varied from one series to another.

^{14}C-Oxalate Deposition

Inhibition of CaOx deposition in each sample was tested using radiolabelled oxalate (Doyle et al. 1995). Each urine sample (30 mL) was added to a designated soda-lime glass flask (to prevent crystals from adhering to the walls of the flask) and incubated in a shaking water bath at 37 °C and 150 rpm for 10 minutes. This was followed by addition of 3.125 μCi ^{14}C-oxalic acid per 100 mL of urine (NEN, Boston, USA). 30 mmol/L of Na_2Ox (10 % v/v) above the previously determined MSL was added to each urine sample, each of which was then incubated for 120 minutes. To stop any further crystal deposit or growth, samples were filtered into concentrated hydrochloric acid (10 % v/v) at 30-minute intervals. Duplicate aliquots (1 mL each) of the acidified urine were added to a 10ml scintillation fluid (Zinsser Analytic, Great Britain) and ^{14}C-oxalate was counted using a scintillation counter (Beckman LS 5000TD Scintillation Counter). The percentage of precipitated ^{14}C-oxalate was determined from the equation (Doyle et al. 1995):

100 - 100x (counts per minute at x min / counts per minute at 0 minute).

CaOx Crystal Aggregation

Inhibition of CaOx crystal aggregation was determined according to the method described by Hess et al. (1989). This method requires the preparation of calcium oxalate monohydrate (COM) and/or calcium oxalate dihydrate (COD) crystals.

(a) Preparation of calcium oxalate monohydrate (COM) crystals

Before the aggregation experiment was conducted, COM crystals were prepared following the method of Pak et al. (1975). Equal volumes of 100 mmol/L $CaCl_2$ and 100 mmol/L Na_2Ox solutions were mixed at a constant rate of 1 mL/min using a peristaltic pump (Gilson, France). The mixture was then stirred at 6 °C for one week. Thereafter, the crystals were filtered through 0.22 μm filters and dried at 37 °C for one hour.

(b) Preparation of calcium oxalate dihydrate (COD) crystals.

COD crystals were prepared according to the method of Brown et al. (1989) with some modifications to the incubation temperature, pH and final volume. A solution containing 1.398 g (38.5 mmol/L) of sodium citrate, 1.423 g (46.2 mmol/L) of magnesium sulphate and 2.403 g (255 mmol/L) of potassium chloride in 250 mL dH_2O was prepared and mixed with 0.920 g (25 mmol/L) calcium chloride solution in 250 mL dH_2O. The resulting mixture was adjusted to pH 6.5 and left to equilibrate at 25 °C in a shaking water bath for 10 minutes. A Na_2Ox solution, 0.216 g (6.40 mmol/L) of Na_2Ox in 250 mL dH_2O was added to the pH adjusted solution and equilibrated at 25 °C for a further 10 minutes. The crystals of the final mixture were collected by filtration through a 0.22 μm pore size, washed with methanol and dried at 37 °C for at least one hour.

(c) Characterization of CaOx crystals.

Crystals prepared in (a) and (b) were characterized using x-ray powder diffraction (XRD). Powdered crystals were packed into aluminium trays and x-ray diffraction patterns were recorded using a Philips PW 1050/80 vertical goniometer in the 12-40 ° 2θ range with CuKα radiation wavelength of 1.5418 Å produced at 40 kV and 25 mA. The observed peaks were assigned by referring to the standard interplanar spacing and relative intensities for COM and COD shown in Table 2.1.

Table 2.1: Interplanar spacings and relative intensities of powder patterns of the two hydrates of CaOx (Sutor and Scheidt 1968).

Calcium oxalate monohydrate (COM)		Calcium oxalate dihydrate (COD)	
d-spacings (Å)	Relative intensity	d-spacings (Å)	Relative intensity
5.93	100	8.70	12
5.79	25	6.31	
4.64	7	6.15	100
4.52	6	4.40	45
3.78		3.89	14
3.76	13	3.67	8
3.65	100	3.58	1
3.00	10	3.38	2
2.97	46	3.15	3
2.91	12	3.09	
2.89	10	3.07	18
2.84	14	2.81	20
2.51	2	2.77	
2.48	30	2.75	85
2.41	5	2.41	14
2.37	2	2.39	14
2.34	90	2.33	10

Figures 2.2 and 2.3 show the x-ray diffraction patterns obtained for CaOx crystals prepared using the methods of Pak et al. (1975) and Brown et al. (1989), respectively. Comparison with the standard x-ray diffraction data given in Table 2.1 confirms that the crystals are calcium oxalate monohydrate and calcium oxalate dihydrate, respectively.

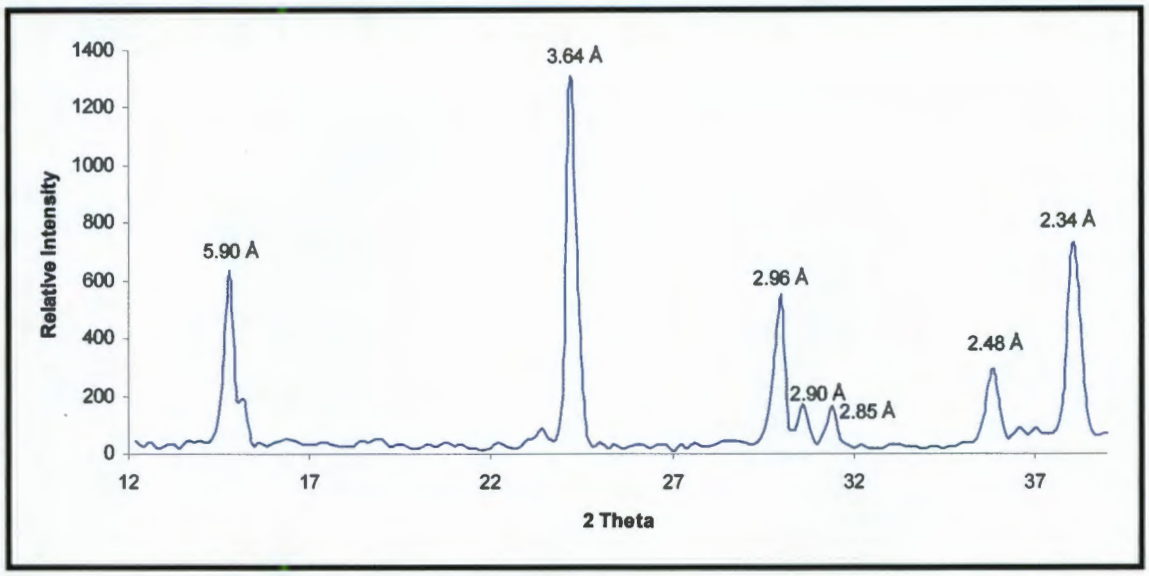


Figure 2.2: XRD pattern of CaOx crystals prepared using the method of Pak et al. (1975).

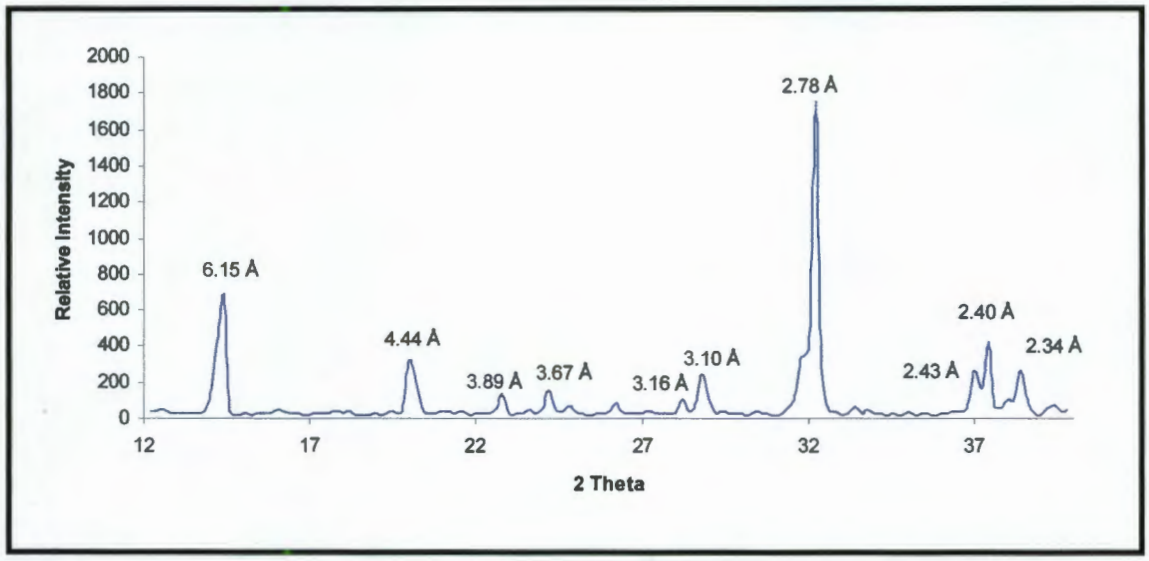


Figure 2.3: XRD pattern of CaOx crystals prepared using the method of Brown et al. (1989).

(d) Determination of COM and COD crystal aggregation inhibition

The COM and/or COD crystals (0.8 mg/mL) were added separately in a buffer containing 10 mmol/L Tris-aminomethane (Tris).HCl and 90 mmol/L NaCl, pH 7.2 (Hess et al. 1989) resulting in a crystal slurry. The crystal slurry was equilibrated overnight at 37 °C with constant stirring at 1100 rpm. The equilibrated slurry was mixed with urine at a ratio of 4:1 (1600 µL: 400µL) or it was mixed with the test protein at a final concentration of 0.5 mg/L and further equilibrated for 2 hours at 1100 rpm. This 4:1 ratio was chosen because the study by Hess et al. (1989) showed that the higher the urine concentration relative to the crystal slurry, the less the inhibition of aggregation. The absorbance of the equilibrated mixture was monitored at 620 nm and 37 °C for 9 minutes using a spectrophotometer (Spectronic Unicam, England). Crystal aggregation was induced by slow stirring at 1100 rpm until equilibrium had been reached which was demonstrated by a constant absorbance. Stirring was then stopped and a decrease in absorbance was monitored indicating the sedimentation rate of the COM/COD crystals. The slope of the linear decrease in absorbance of the slurry (labelled "absorbance slope" in Figure 2.4) indicated the degree of crystal aggregation of COM/COD crystals in the presence of urine. The percentage inhibition of aggregation by the urine sample was determined from the equation (Hess et al. 1989):

1 – [St (slope of the test sample)/Sc (slope of the COM/COD slurry without urine)] X 100.

A typical experimental curve is shown in Figure 2.4.

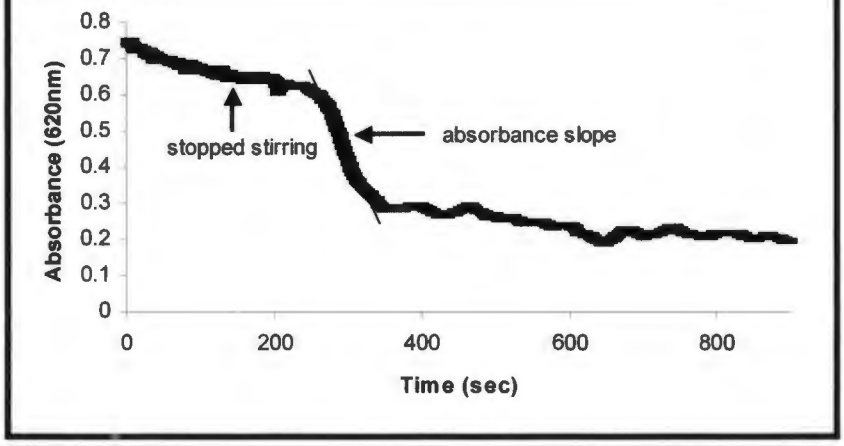


Figure 2.4: A typical CaOx crystal sedimentation plot of absorbance vs. time.

CaOx Crystal Nucleation

Inhibition of CaOx crystal nucleation was determined using the method developed by Hess et al. (1995). Stock solutions of 8.5 mmol/L of calcium chloride, $CaCl_2$ and Na_2Ox (1.0 mmol/L) each containing 200 mmol/L sodium chloride and 10 mmol/L sodium acetate, were prepared and the pH was adjusted to pH 5.7. The stock solutions were filtered through a 0.22 μm filter to remove any debris that might interfere with the spectrophotometric measurements.

The experiment was performed at 37 °C using a circulating water bath. $CaCl_2$ solution (1 mL) was transferred into a 10-mm cuvette placed in a spectrophotometer (Analytikjena, specord 40), regulated at 37 °C and constantly stirred at 500 rpm. 1 mL of the Na_2Ox solution was added to the $CaCl_2$ solution, resulting in final assay concentrations of 4.25 mmol/L calcium and 0.5 mmol/L oxalate, respectively. The automated time course measurement of OD_{620} was performed after the addition of Na_2Ox solution to the $CaCl_2$ solution. These values were recorded every 0.5 seconds for 60 minutes. The maximum slope of increase of OD_{620} with time representing crystal nucleation for the control was termed (S_N). Once equilibrium had been reached, crystals neither nucleated nor grew but there was a progressive decrease of OD_{620}. A typical crystal nucleation curve is shown in Figure 2.5.

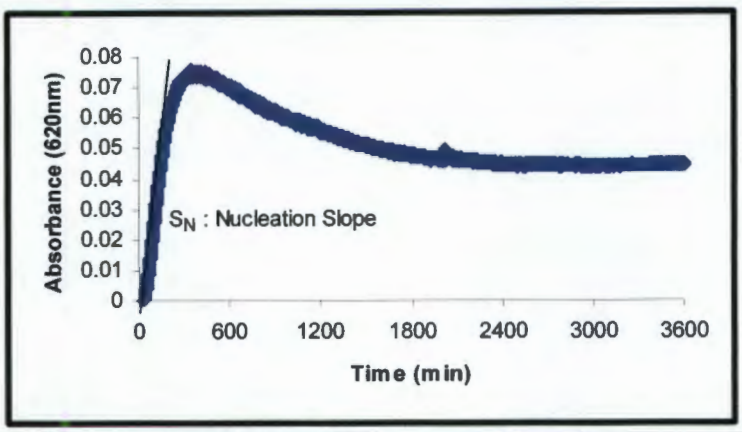


Figure 2.5: A typical plot of absorbance vs. time for the determination of CaOx nucleation and aggregation assay.

This experiment was performed in the presence of (i) urine, (ii) commercially available osteopontin or CME and (iii) urine + osteopontin. The final concentration of 0.5 mg/L of protein was used. This concentration corresponds to that which achieved maximum inhibition of crystal aggregation in the study reported by Doyle et al. (1995). The urine samples were tested at a ratio of 800 µL of $CaCl_2$: 400 µL of urine: 800 µL of Na_2Ox. OD_{620} was recorded every 0.5 seconds over 60 minutes. This urine concentration of 400 µL urine/ 2mL total solution corresponds to that which was used for the aggregation experiment described on the previous page and it achieved the highest inhibition of crystal aggregation as reported by Hess et al. (1989). The maximum slope increase (positive slope) of OD_{620} obtained with time was termed (S_N) and used for the calculation of nucleation inhibition. The percentage inhibition of nucleation by the test sample was determined from the following equation:

$$\% N_{IN} = 100 (1 - S_T / S_N)$$

N_{IN} = Nucleation inhibition
S_T = Slope in the presence of test sample
S_N = Slope of nucleation of the control without test sample

CaOx crystal Zeta Potential

(a) The concept of zeta potential

As explained by several authors, aggregation of particles in solution is governed by an interplay of several basic forces which either have aggregating or disaggregating effects (Finlayson 1978; Robertson et al. 1981; Hess 1991). Two forces which favour aggregation are the attractive van der Waals forces and viscous binding. The main force which favours disaggregation is the zeta potential (Hess and Kok 1996). Zeta potential is the repulsive electrical potential that exists at the interface between a layer of chemically adsorbed ions on a crystal surface (solid phase) and its surrounding solution (continuous phase) (Scurr and Robertson 1986). In water COM crystals have a zeta potential of about +20 mV (Curreri et al. 1979). In urine, the zeta potential becomes more negative because of the adsorption of negatively charged urinary molecules such as citrate, pyrophosphate, and acidic macromolecules (Finlayson et al. 1984). It has been shown that highly anionic macromolecules such as GAGS, THP and nephrocalcin can induce zeta potentials between -15 and -40 mV (Scurr and Robertson 1986; Hess et al. (1989). These authors regard zeta potential as an indicator of the potency of a compound to inhibit aggregation by virtue of its repulsive negative charge. The greater the magnitude of the negative charge, the greater is the inhibition.

(b) Preparation of COM and COD crystal slurries for zeta potential measurements

The slurries were prepared following the method of Hess et al. (1989). A 10 mmol/L sodium acetate buffer was prepared by mixing 10 mmol/L of sodium acetate with 270 mmol/L of sodium chloride. The pH was adjusted to 5.7 by addition of sodium hydroxide. Crystal slurries were then prepared by addition of 0.03 mg/mL of COM or COD crystals to the pH-adjusted sodium acetate buffer. The slurries were equilibrated overnight with constant stirring at 1100 rpm and 25 °C.

(c) Measurement of zeta potential

The equilibrated slurries were mixed with either CME (from black and white subjects) or commercially available osteopontin (at a final concentration of 0.5 mg/L) and further equilibrated for 2 hrs at 1100 rpm. The zeta potential of the slurries without the proteins was measured in triplicate using a Zetasizer Nanoseries (Malvern Instruments, England) and used as a control. Thereafter, the zeta potential of the crystals in the presence of CME or osteopontin (triplicate measurements) was also determined.

CHAPTER THREE

INHIBITORY ACTIVITY OF URINARY MACROMOLECULES (MW > 10 kDa) FROM BLACK AND WHITE SUBJECTS

3.1 INTRODUCTION

Robertson and his co-workers reported that CaOx urolithiasias may be the net result of an imbalance between the two opposing influences of urinary supersaturation and urinary inhibitors (Robertson et al. 1976). Several studies (Robertson et al. 1968; Coe et al. 1980; Drach et al. 1980) have reported a deficiency in the inhibitory activity of urine from stone-formers.

Since urine physico-chemical and relative supersaturation analyses alone do not distinguish between healthy individuals and stone-formers (Tiselius et al. 1995), and indeed between South Africa's two population groups (Rodgers 2006), potential differences in the inhibitory properties of the urinary macromolecules in both race groups warrants investigation. Studies suggest that most of urine's inhibitory activity with respect to CaOx resides in macromolecules which are active at submicromolecular concentrations (Nakagawa et al. 1983; Atmani et al. 1996; Ryall 1997). Many of these macromolecules have been found in the protein matrix that is a part of all calcium stones (Worcester 1996). Most previous workers have concluded that macromolecules greater than 10 kDa, considered altogether, exert an inhibitory effect on CaOx crystal growth and aggregation in whole human urine (Edyvane et al. 1987; Koide et al. 1990; Sorensen et al. 1995; Tiselius and Fornander 1990; Bek-Jensen and Tiselius 1991; Ryall et al. 1991; Ebisumo et al. 1999). Nevertheless, some macromolecules such as Tamm-Horsfall Protein (THP) have been reported to promote CaOx crystallization (Hallson and Rose 1977; Grover et al. 1990).

Since urinary macromolecules greater than 10 kDa have not been previously studied in the context of the South African black and white paradox, the study described in this chapter was undertaken with this purpose in mind.

3.2 AIMS AND OBJECTIVES

- To analyze 24 hour urine samples from black and white healthy males for routine physico-chemical properties.
- To investigate the relative inhibitory roles of urinary macromolecules in concert (> 10 kDa) from black and white healthy males towards CaOx crystallization by determining CaOx metastable limits, ^{14}C-oxalate deposition kinetics and CaOx crystal aggregation.

3.3 MATERIALS AND METHODS

3.3.1 Urine collection and treatment and physichochemical properties.

24 hour urine samples were obtained from 10 black (B) and 10 white (W) healthy males. Each sample was tested, treated and analyzed as described in Chapter 2. After filtration (0.75 μm pre-filter and 0.45 μm nitrocellulose filter), a portion of each prefiltered sample (P) was retained for further experiments and the rest was ultrafiltered using a 10 kDa membrane cut-off (Millipore® TM, Minitan System, USA) to produce a concentrate (C) and an ultrafiltrate (UF). The crystallization properties of each of these three fractions were tested in various experiments, as follows.

3.3.2 Crystallization Experiments

CaOx Metastable Limit (MSL)

The CaOx MSL of each urine fraction (n = 10) was determined following the method described by Ryall and co-workers (Ryall et al. 1985) as described in Chapter 2 .

Scanning Electron Microscopy (SEM)

Once the MSL of each urine fraction had been determined, crystallization was induced and the deposited crystals were viewed using SEM as described in Chapter 2. All the fractions in 5 samples from each group were examined.

Inhibition of ^{14}C-Oxalate Deposition

Inhibition of CaOx deposition in each urine fraction from all 10 subjects in each group was tested using radiolabelled oxalate as described in Chapter 2 (Doyle et al. 1995).

Inhibition of CaOx Crystal Aggregation

The previously described COM crystal slurry was equilibrated overnight at 37 °C with constant stirring at 1100 rpm as previously described in Chapter 2. Portions of the equilibrated slurry were individually mixed with each urine fraction from all 10 subjects from each group at a ratio of 4:1 slurry to urine and further equilibrated for 2 hours at 1100 rpm. The absorbance of the equilibrated mixture was monitored at 620 nm and 37 °C for 9 minutes using a spectrophotometer (Spectronic Unicam, England) and the percentage inhibition of aggregation by each urine fraction was calculated as previously described (Chapter 2, page 43).

3.4 RESULTS

3.4.1 Urine composition and physicochemical parameters of 24 hour samples

The mean composition and physicochemical parameters of individual ultrafiltered 24 hour urine samples from black and white subjects are shown in Table 3.1. Individual parameters for each sample from both groups are shown in Appendix 1, Tables 1.1 and 1.2. Parameters that showed a significant difference ($p \leq 0.05$) between the two groups are denoted with an asterisk. Urinary citrate, magnesium, uric acid and RS of CaOx and RS of uric acid were significantly lower in samples from black subjects than those from white subjects.

Table 3.1: Mean composition and physicochemical parameters (± SE) of 24 hour urine samples from black and white subjects (n = 10B, n = 10W).

Parameters	B ± SE	W ± SE	p-value
pH	6.37 ± 0.130	6.21 ± 0.130	0.371
Volume (mL/24hr)	1526 ± 157	1272 ± 157	0.272
Calcium (mmol/24hr)	3.31 ± 0.480	4.33 ± 0.480	0.181
Citrate (mmol/24hr)	1.43 ± 0.180	2.26 ± 0.180	*0.003
Chloride (mmol/24hr)	127 ± 12.2	126 ± 12.2	0.958
Creatinine (mmol/24hr)	12.2 ± 1.46	16.5 ± 1.46	*0.052
Magnesium (mmol/24hr)	2.26 ± 0.480	3.37 ± 0.480	*0.034
Oxalate (mmol/24hr)	0.160 ± 0.0200	0.150 ± 0.0200	0.609
Phosphate mmol/24hr)	16.3 ± 3.76	37.9 ± 3.76	*0.001
Potassium (mmol/24hr)	145 ± 39.2	48.7± 39.2	0.101
Sodium (mmol/24hr)	74.0 ± 17.9	95.7 ± 17.9	0.409
Sulphate (mmol/24hr)	12.2 ± 1.63	19.0 ± 1.63	*0.011
Uric acid (mmol/24hr)	2.65 ± 0.410	4.09 ± 0.410	*0.023
RS Brushite	1.79 ± 0.640	2.00 ± 0.640	0.813
RS CaOx	0.33 ± 0.350	2.47 ± 0.350	*0.001
RS Uric acid	0.74 ± 0.310	1.69 ± 0.310	*0.040

**Difference is statistically significant: $p \leq 0.05$, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

3.4.2 Crystallization Experiments

CaOx Metastable Limit

Tables and plots for individual urines are given in Appendix 1, Tables 1.3 and 1.4 as well as Figures 1.1 and 1.2, respectively. In order to compare these, particle counts for the individual experiments were averaged, yielding the mean plots shown in Figures 3.1 (a) and (b) below. However, it is recognized that such an approach might not necessarily give absolute values for the MSL. Nevertheless, the approach allows a semi-quantitative comparison to be made between black and white subjects.

The concentration that corresponds to the MSL of each fraction is denoted by a colour coded arrow. All the urine fractions from white subjects had the same MSL (0.015 mol/L). The MSL for the urine fractions from black subjects followed the trend C > UF > P, where C: concentrate, UF: ultrafiltrate and P: prefiltrate with the corresponding values of 0.060, 0.045, and 0.030 mol/L, respectively. These values show that urines from white subjects for all the three fractions have significantly lower MSL (0.015 mol/L; $p < 0.05$) than urine from black subjects (range: 0.030 – 0.060 mol/L; $p < 0.05$).

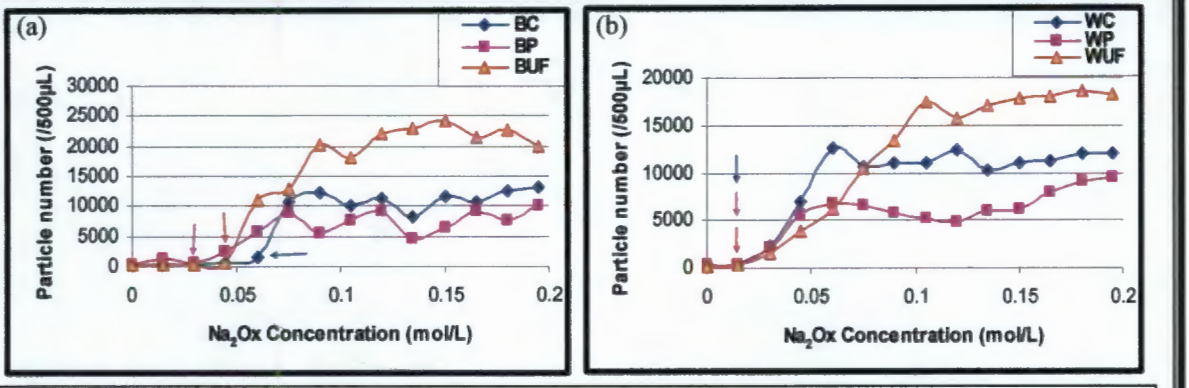


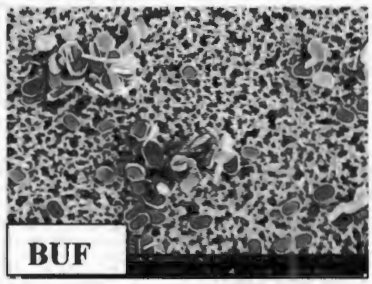
Figure 3.1: Mean metastable limit (MSL) plots for three urine fractions: ultrafiltrate (UF); prefiltrate (P); and concentrate (C) from (a) 10 black (B) and (b) 10 white (W) male subjects.

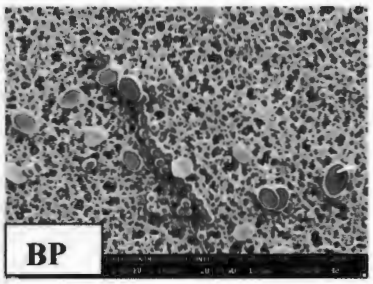
Comment

The significantly higher MSL of all the fractions from black subjects is an indication of resistance to CaOx crystal formation in urine from this group. Moreover, the results clearly demonstrate that the various fractions of the urine from black subjects have different inhibitory capacities themselves whereas those from white subjects are indistinguishable in this regard. Of even greater interest, is the observation that while removal of macromolecules > 10 kDa in whites had no effect on the MSL, concentrating the macromolecules in the urine from black subjects caused the MSL to increase.

Scanning Electron Microscopy (SEM)

Representative scanning electron micrographs of crystals obtained after the induction of CaOx crystallization in urine fractions from black and white subjects are shown in Figures 3.2 and 3.3, respectively. Crystals deposited in urine fractions from black subjects were mainly COM compared to COD from white subjects. Generally, the degree of aggregation was greater in all the fractions from white subjects compared to those from black subjects. In both groups, aggregation decreased in the sequence UF < P < C. It was observed that crystals in all the fractions from black subjects were smaller than those in white subjects.





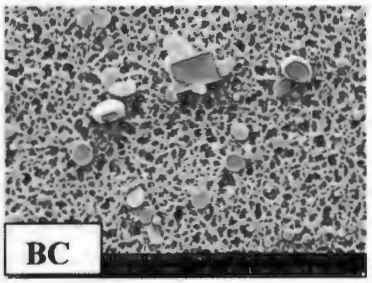
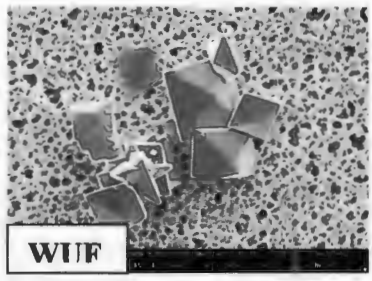
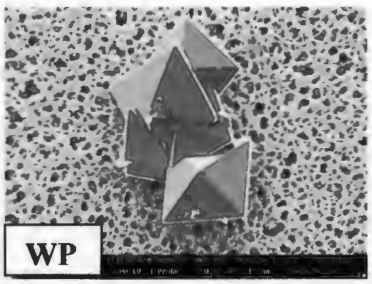


Figure 3.2: Scanning electron micrographs of crystals induced from the three urine fractions from black subjects: ultrafiltrate (BUF); prefiltrate (BP); concentrate (BC) at 10 K magnification.





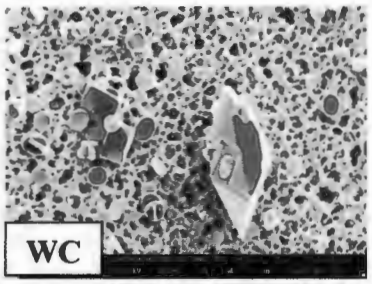


Figure 3.3: Scanning electron micrographs of crystals induced from the three urine fractions from white subjects, ultrafiltrate (WUF); prefiltrate (WP); and concentrate at 10 K magnification.

Comment

These results suggest that urinary macromolecules (MW > 10 kDa) in black subjects are more effective inhibitors of CaOx crystal growth and aggregation than those in the urine of their white compatriots. The fewer number of individual crystals demonstrated by the micrographs in the concentrate (C) and the prefiltrate (P) of samples from black subjects suggests more effective inhibition of crystal nucleation and aggregation in this group.

^{14}C-Oxalate Deposition

The mean percentage of ^{14}C-oxalate deposited by each urine fraction from black and white subjects is shown in Figures 3.4 (a) and (b), respectively. Individual graphs for each urine fraction are shown in Appendix 1, Figures 1.3 and 1.4.

Both figures demonstrate that the percentage deposition follows the trend concentrate < prefiltrate < ultrafiltrate (C < P < UF). Deposition induced by all urine fractions from white subjects (52 %, 40 % and 35 % for WC, WP and WUF, respectively) was slightly higher compared to those from black subjects (48 %, 32 % and 31 % for BC, BP and BUF, respectively) but these differences were not statistically significant.

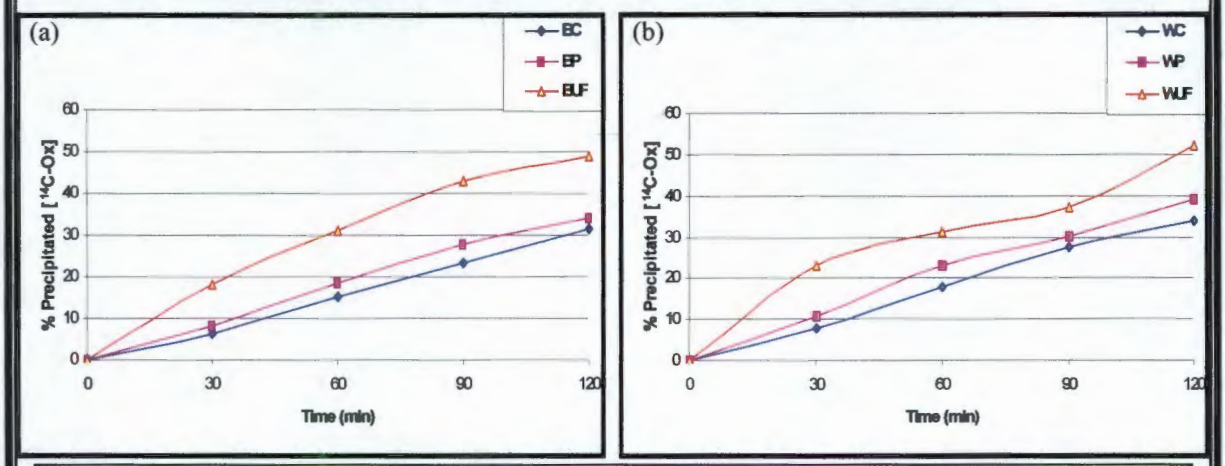


Figure 3.4: Mean percentage [^{14}C]-Oxalate deposited in three urine fractions: ultrafiltrate (UF); prefiltrate (P); and concentrate (C) from 10 black (a) and 10 white (b) male subjects.

Comment

The similar trend C < P < UF demonstrated by both groups indicates that urinary macromolecules in both race groups are inhibitors of CaOx crystal deposition. It is evident from these results that there is no distinction that can be made between the relative efficacies of the macromolecules from the two groups in this regard. Nonetheless, all the fractions from black subjects displayed a trend which suggested a slightly stronger inhibition of CaOx deposition than that from the white subjects.

CaOx crystal aggregation inhibition

Figure 3.5 shows the mean plots of absorbance vs. time from which the sedimentation rates of a COM crystal slurry were determined before and after the addition of the urine fractions at a ratio of 4:1 of COM slurry to urine, from both black and white male subjects. Table 3.1 shows the average slopes obtained and the corresponding calculated percentage inhibition demonstrated by each of the urine fractions.

Figure 1.5 in Appendix 1 shows the individual COM plots from which the mean COM slurry slopes were calculated. Individual plots and percentage inhibition values are shown in Appendix 1, Figures 1.6 and 1.7 and Tables 1.5 and 1.7 for urine fractions from black and white subjects respectively.

It was noted that the percentage inhibition by the concentrate and prefiltrate was the same within a particular race group (83 % for blacks and 76 % for whites). It was also noted that the extent of inhibition was higher in the black group (range 76 % – 83 %) than in the white group (72 % - 76 %). However, these differences were not statistically significant.

Table 3.2: Mean slopes of absorbance vs. time graphs, R^2 and percentage inhibition of aggregation (I_A %) induced by each urine fraction from black subjects.

Sample	Slope X -10^{-4} (OD/sec); (R^2)	I_A % (p-values , wrt COM)	p-values
COM	29; (0.955)		
COM + BC	5.0; (0.962)	83 ($p < 0.05$)	
COM + BP	5.0; (0.991)	83 ($p < 0.05$)	
COM + BUF	7.0; (0.995)	76 ($p < 0.05$)	
Comparisons			
BUF vs. BC			**0.234**
***BUF vs. BP**			***0.016**
BC vs. BP			**0.602**
COM + WC	7.0; (0.971)	76 ($p < 0.05$)	
COM + WP	7.0; (0.990)	76 ($p < 0.05$)	
COM + WUF	8.0; (0.983)	72 ($p < 0.05$)	
Comparisons			
WUF vs. WC			**0.447**
WUF vs. WP			**0.179**
WC vs. WP			**0.444**
Comparisons			
BC vs. WC			**0.248**
BP vs. WP			**0.106**
BUF vs. WUF			**0.503**

** indicates that the difference is statistically significant: $p \leq 0.05$*

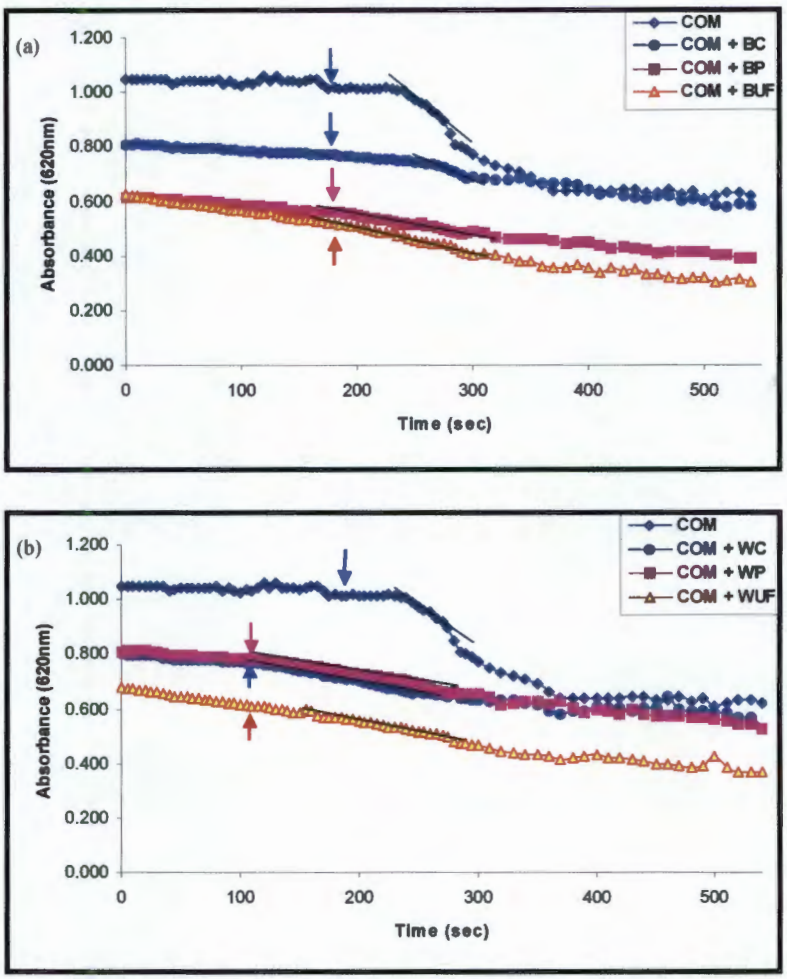


Figure 3.5: Mean COM crystal sedimentation plot of absorbance vs. time before and after the addition of urine fractions from (a) black and (b) white subjects at a ratio of 4:1 slurry to urine. The arrows show the time at which stirring was stopped and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Comment

These results demonstrate a higher aggregation inhibition by the urine concentrates and prefiltrates than the ultrafiltrates from both race groups. This indicates that the urinary macromolecules from both race groups are strong inhibitors of CaOx crystal aggregation. The same extent of aggregation inhibition by the concentrate and prefiltrate is surprising since it suggests that the efficacy of the macromolecules to achieve inhibition of aggregation is independent of concentration. Indeed, no significant difference was observed between samples from black and white subjects. However, a significantly higher aggregation inhibition was noted in the concentrates from black subjects compared to the prefiltrates from the same group.

3.5 DISCUSSION

Urine composition data demonstrated that citrate, magnesium, phosphate and uric acid were significantly lower in urine samples from black subjects. Relative supersaturation of CaOx and uric acid (UA) were also significantly lower in this group. All of these physiochemical risk factors are in agreement with the empirically observed lower stone incidence in black subjects relative to their white compatriots. However, all of these values *lie within the normal range*. Therefore, they alone cannot explain the virtual absence of stones in this race group.

Normal urine itself retards and inhibits crystallization (Pak et al. 1976; Coe et al. 1991) and thus it is possible that the black population's urine is superior to whites in this regard. Indeed, the present study demonstrated this. MSLs showed that urine samples from white subjects have a relatively lower MSL compared to urine from black subjects and that this was independent of the composition of the urine with respect to macromolecules. This is in line with the results reported by Ryall et al. (1986), who demonstrated that stone formers have lower MSLs than healthy controls. As indicated earlier, the presence of macromolecules > 10 kDa in the urine from black subjects induced an increase in the MSL, whereas it had no effect on the MSL of the urine from their white counterparts. This provides compelling evidence that the macromolecules in the urine of blacks are superior inhibitors of CaOx crystallization.

In both race groups, ^{14}C-oxalate deposition followed the same trend for all the three urine fractions, namely C < P < UF. These results indicate that concentrated urine (which contains the greatest proportion of macromolecules) is a potent inhibitor of CaOx deposition in both race groups.

Spectrophotometric data demonstrated a higher inhibition of aggregation by the urine concentrates than the ultrafiltrates from both race groups. Of great interest is that the concentrate and the prefiltrate fractions from both race groups inhibited crystal aggregation to the same extent. As mentioned earlier, this suggests that inhibition by these macromolecules proceeds independently of concentration. However, it also indicates synergism between macromolecules of all sizes in preventing CaOx crystallization. Comparing the two race groups, the inhibition induced by urine samples from the black group was 8 % higher than that of the white group. Even though this difference is relatively small, this result is in

agreement with that demonstrated by Asplin (1999) and Tiselius et al. (1995) when comparing the CaOx aggregation inhibition by normal men and stone formers. It is also noteworthy that when macromolecules > 10 kDa were removed, inhibition of CaOx aggregation decreased, thereby clearly indicating that this crystallization mechanism is controlled by macromolecules whose size exceeds this lower limit.

The aggregates of large COD crystals observed in urine fractions from white subjects relative to the free COM crystals deposited in fractions from black subjects are in line with the results reported by Robertson et al. (1968) and Dent (1971) who observed the identical differences in crystal type in the urine of stone formers and that of healthy men. This evidence supports the notion that normal urine contains some inhibitors of CaOx crystal aggregation (Robertson and Peacock 1972). Moreover, the precipitation of COD crystals compared to COM crystals is considered to be of great pathological importance since the former are less adherent to renal epithelial cells (Wesson et al. 1998; Cerini et al. 1999).

The results reported in this chapter have shown that filtration removes urinary macromolecules which in turn increases CaOx crystallization in both race groups. However, smaller crystals and a lower degree of crystal aggregation together with greater inhibition of crystal deposition and CaOx aggregation in samples from black subjects compared to their white fellow citizens suggest more effective inhibition in the former group. This demonstrates the inhibitory role of macromolecules in the black population group. Therefore macromolecular inhibitors of CaOx deposition and aggregation may play a role in contributing towards stone rarity in black subjects.

CHAPTER FOUR

INHIBITORY EFFECT OF URINARY PROTEINS ASSOCIATED WITH CALCIUM OXALATE DIHYDRATE (COD) CRYSTALS IN THE URINE OF BLACK AND WHITE SOUTH AFRICAN MALES

4.1 INTRODUCTION

Approximately 2-5 % of total dry weight of CaOx renal stones is composed of the organic matrix (Boyce and Garvey 1956; Boyce and King 1959; Boyce 1968; Warpehoski et al. 1981; Stalcholy and Goldberg 1985). As initially analyzed by King and Boyce (1959), about 64% of this organic matrix is protein. In their experimental model, Khan and Hackett (1987) demonstrated that the organic material becomes intimately associated with crystals during the early stages of their development. Following these findings, the association of urinary macromolecules with CaOx crystals has been extensively studied (Doyle et al. 1991 and 1995; Sorensen et al. 1995; Atmani et al. 1996; Honda et al. 1997; Atmani and Khan 2002; Webber et al. 2003; Ryall et al. 2005). The major finding of these studies is that proteins are selectively incorporated into CaOx crystals.

Numerous studies have been conducted to identify and characterize the proteins incorporated into the crystals matrix extract (Atmani et al. 1996; Honda et al. 1997; Ryall et al. 2000; Atmani and Khan 2002) and to test their inhibitory properties against CaOx urolithiasis (Shiraga et al. 1992; Sorensen et al. 1995; Doyle et al. 1996; Webber et al. 2002). These studies have focused on the inhibitory properties of the matrix extract protein incorporated in CaOx crystals without attention being directed on either COM or COD.

As described in Chapter 1, the black South African population has a relatively lower urinary calcium concentration compared to their white counterparts (Modlin et al. 1967; Whalley et al. 1998; Rodgers et al. 2002). Lower urinary calcium concentrations favour the formation of COM crystals while higher concentrations favour COD crystal formation (Burns and Finlayson 1980). Webber et al. (2002) have shown that urinary crystals precipitated from black subjects are mainly composed of COM compared to COD precipitated from white subjects. Considering the relatively lower urinary calcium concentration of blacks, this is not

surprising. The two main proteins in the organic matrix of CaOx crystals have been reported to be osteopontin (OPN) (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005) and UPTF1 (Doyle et al. 1995; Ryall et al. 2000; Webber et al. 2002 and 2003; Ryall et al. 2005). UPTFI is the principal intracrystalline protein in COM crystals (Doyle et al. 1995; Webber et al. 2002 and 2003; Ryall et al. 2005) and OPN in COD crystals (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005). COM and COD crystals can be selectively precipitated from urine by adjusting the calcium concentration (Burns and Finlayson 1980).

The study described in this chapter addresses for the first time, the inhibitory properties of the matrix extract protein included in COD crystals from the urine of black and white South African subjects. COD instead of COM was selected for this study because the latter has already been investigated in this context (Durrbaum et al. 2001; Webber et al. 2002 and 2003; Rodgers et al. 2006). Since there is a high abundance of osteopontin in COD crystals (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005) compared to other proteins, it was anticipated that by focusing attention on the COD crystal matrix extract from the two race groups, insights into the possible inhibitory role of this urinary protein in the context of the South African black versus white stone paradox, might be obtained.

4.2. AIMS AND OBJECTIVES

- To precipitate COD crystals in urine samples from black and white healthy subjects.
- To isolate and characterize the crystal matrix extract (CME).
- To semi-quantitatively measure osteopontin (OPN) in CME using Western Blotting.
- To conduct a series of crystallization experiments investigating the possible inhibitory role of COD-CME proteins in ultrafiltered urine of black and white healthy male subjects.
- To repeat the investigation in a cross-over design involving the addition of COD-CME proteins from black subjects in urine samples from whites, and vice versa.

4.3. MATERIALS AND METHODS

4.3.*1* Urine collection and treatment

Individual 24 hour urine samples from 35 different black (B) and 35 different white (W) healthy males were obtained without preservatives and were tested and filtered as previously described in Chapter 2. Seven pools comprising 1.5 L from each of 5 urine samples were then constructed. Urine compositional analyses were not performed.

4.3.*2* Preparation and Isolation of COD-CME

The calcium concentration of each pooled urine sample was measured using atomic absorption spectroscopy (Fernandez and Khan 1971). The CME was prepared from each pooled urine sample following a protocol described by Doyle et al. (1991). In this method, the urines were incubated at 37 °C in a shaking water bath (Labcon Johannesburg) and 1 mol/L $CaCl_2$ was added to a final concentration of 12 mmol/L. The CaOx MSL of the calcium-adjusted urine was measured using the method of Ryall et al. (1985). 30 mmol/L Na_2Ox was added in excess of the previously determined MSL at 0, 1, 2, 3 hours to induce COD crystallization. Crystals were collected by filtration through 0.22 μm filters and washed with distilled water, dried and analyzed by x-ray powder diffraction (Sutor and Scheidt 1968). The collected crystals were then demineralized in 0.25 mmol/L EDTA pH 8.0 (250 mmol/L per gram of crystals) yielding the calcium oxalate dihydrate crystal matrix extract (COD-CME), henceforth referred to as CME. This was desalted by extensive dialysis against deionized water at 4 °C for 48 hours using a dialysis membrane cut-off of 10 kDa. The resulting CMEs from the seven pools were combined and stored at -20 °C for further use.

4.3.*3* Characterization of COD-CME

SDS-PAGE

1 mg of COD-CME from each group was dissolved in 10 μL of water. 2 μL of 5 X SDS reduction buffer was added to the samples and boiled at 100 °C for 5 minutes. Once the samples had cooled down, a low molecular marker standard (Bio-Rad) was added and they were subjected to 10 % sodium dodecyl sulfate polyacryalamide gel electrophoresis (SDS-

PAGE) and 3 % stacking gel. The gels were electrophoresed using Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, California, USA) at 25 mA per gel for 1 hour. The electrophoresed gels were stained with Coomassie blue (0.05 % Coomassie, 50 % methanol and 7 % acetic acid) for 1 hour, for detection of protein bands followed by destaining with 25 % ethanol and 7 % acetic for 30 minutes or blotted in a blotting buffer for immunodetection of osteopontin (OPN).

Western Blotting for OPN

The electrophoresed gel together with a nitrocellulose membrane was equilibrated in a blotting buffer for 15 minutes (Appendix 2.1). The proteins from the gel were transferred onto a nitrocellulose membrane by running it at 100 V or 300 mA for 60 minutes. The membrane was then blocked for 1 hour in blocking buffer (Appendix 2.1) followed by 3 X 15 minutes washes in blocking buffer. This was followed by incubation with the primary antibody for 90 minutes. The primary antibody used was a rabbit polyclonal osteopontin antibody (Abcam, Cambridge, England) at a dilution of 1:500. After 3 X 15 minutes washes in blocking buffer the blot was incubated with the secondary rabbit antibody (Amersham Biosciences, New Jersey, USA) at a dilution of 1:2000 for 60 minutes. A third 3 X 15 minutes wash was carried out prior to detection of OPN with 4-chloro-1-naphthol (Sigma-Aldrich) for 20 minutes.

Bradford Assay

The Bradford assay, (Bradford 1976) was performed using bovine serum albumin (BSA) standards (Bio-Rad) to determine the total protein concentration in crystal matrix extracts from black and white subjects, henceforth referred to as BCME and WCME respectively. Triplicate standard solutions of BSA (Bio-Rad) were prepared and measured at 595 nm against the standard blank using a UV-vis spectrophotometer (Spectrophotometer, Anthelie, France). A standard curve was plotted from which the protein concentrations were calculated.

4.3.4 Crystallization Experiments

The effect of the CME from both race groups on particle formation kinetics, [^{14}C]-oxalate deposition kinetics, aggregation and nucleation of CaOx crystals was determined at a final concentration of 0.5 mg/L in 7 different pools (independent to those described in 4.3.1) of 5 freshly collected 24 hour urine samples (200 mL each sample) from black and white healthy males. This concentration corresponds to that which achieved maximum inhibition of crystal aggregation in the study reported by Doyle et al. (1995). The pooled samples were prefiltered through 0.75 μm filters (Macherey-Nagel; GmBH and Co., Germany), followed by further filtration through a 0.45 μm nitrocellulose filter (Sartorius AG, Germany) and then ultrafiltered through a 10 kDa cut-off membrane (Millipore Corporation, Bedford; USA). The ultrafiltrate was used for the crystallization experiments.

CaOx Metastable Limit (MSL)

The MSL of the pooled ultrafiltrate (UF) samples was determined as described in Chapter 2 following the method of Ryall et al. (1985). The MSL was not determined in the presence of the protein because it has been reported that it does not have an effect on the metastable limit (Doyle et al. 1995). In all experiments, the ultrafiltrate was used as the control.

Cross-over design experiment

For the kinetics and [^{14}C]-oxalate deposition experiments, a cross-over approach was adopted (Craig et al. 1999). This permitted the effect of each protein to be tested in its parent urine and in the urine from the other race group. Thus, WCME and BCME were each independently tested in BUF and WUF (Figure 4.1)

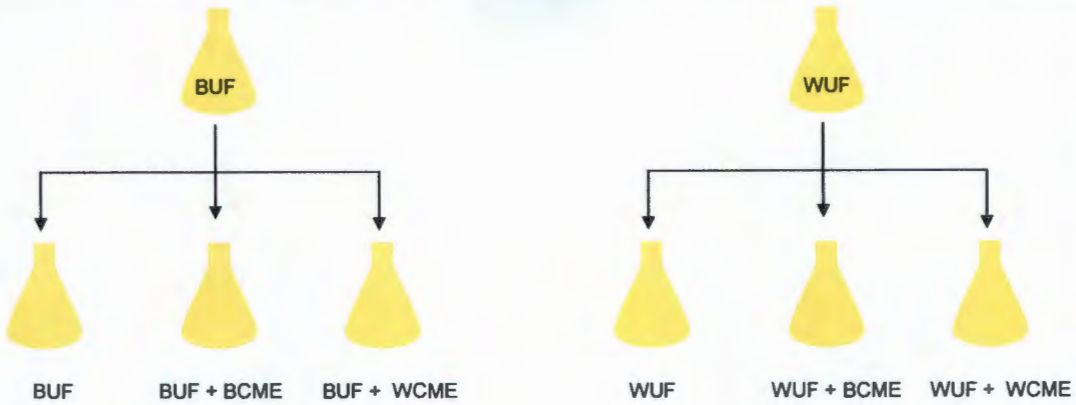


Figure 4.1: Cross-over design crystallization experiment.

Effect of CME on particle number, volume and size

Three conical flasks, each containing 50 mL of BUF or WUF, were set up as shown in Figure 4.1. The first flask served as a control and the proteins were added to the other flasks. Once the MSL had been determined (in BUF and WUF), 10 % (v/v) of Na_2Ox at a concentration of 30 mmol/L above the MSL was added to each flask and incubated at 37 °C for 2 hours in a shaking water bath set at 100 rpm (Labcon, Johannesburg). A zero time analysis was performed before the addition of Na_2Ox and the reaction was monitored as previously described in Chapter 2 using a Coulter Multisizer I (Coulter Electronics Ltd., England).

Effect of CME on [^{14}C]-oxalate deposition

This experiment was conducted as described in Chapter 2. Three sets of soda-glass flasks each containing 30 mL of BUF or WUF were set up as shown in Figure 4.1. The first flask served as the control and once the proteins had been added to the other flasks, the reaction was continued as previously described in Chapter 2. The percentage of precipitated ^{14}C-oxalate by each protein was determined.

Effect of CME on COM and COD crystal aggregation inhibition

For the aggregation experiment, the COM and COD crystals were treated the same way (but separately) as in Chapter 2 (Hess et al. 1989). After an overnight equilibration of COM crystals in 10 mmol/L Tris-aminomethane (Tris).HCl and 90 mmol/L NaCl, pH 7.2 buffer at 37 °C with constant stirring at 1100 rpm, the resulting slurry was then mixed with UF and CME at a slurry–to-urine ratio of 4:1 (1600 µL: 400 µL) and a total protein concentration of 0.5 mg/L and equilibrated for 2 hours at 1100 rpm. The absorbance of the equilibrated mixture was monitored at 620 nm at 37 °C for 15 minutes and inhibition of COM crystal aggregation was determined as described in Chapter 2. The protocol was then repeated using COD crystals except that the inhibition was determined in the absence of UF.

Effect of CME on crystal nucleation

The effect of CME on crystal nucleation was determined using the method developed by Hess et al. (1995) as previously described in Chapter 2. This experiment was performed with the protein only at a final concentration of 0.5 mg/L using 1 mL $CaCl_2$ and 1 mL Na_2Ox solutions. OD_{620} was recorded every 0.5 seconds over 60 minutes.

4.4 RESULTS

4.4.1 Characterization of CME

XRD was used for the identification of the crystals precipitated from the urine of black and white subjects after adjustment of the calcium concentration to 12 mmol/L. The XRD pattern obtained for crystals from both black and white subjects is shown in Figure 4.2. Interplanar d-spacings for the most prominent reflections are indicated. This pattern corresponds with the standard spectrum for COD (Sutor and Scheidt 1968).

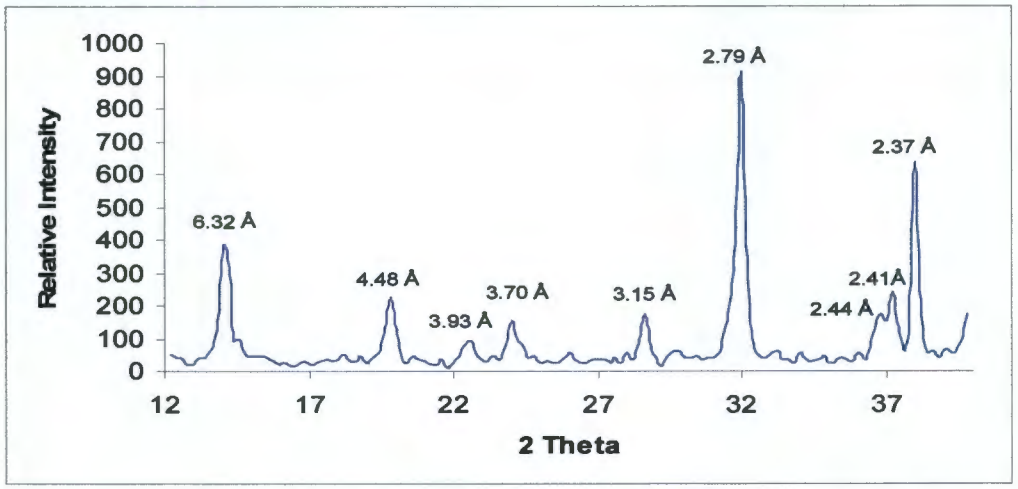


Figure 4.2: XRD pattern of crystals precipitated from B and W urine samples after adjusting Ca concentration to 12 mmol/L.

4.4.2 SDS-PAGE and Western Blotting for OPN

Figure 4.3 shows a Coomassie stained gel of 1 mg of BCME and WCME. Both samples showed bands at 30, 66 and 97 kDa, which might be due to low amounts of UPTF1 (30 kDa), and osteopontin, (66 and 97 KDa). The OPN immunoblot blot is shown in Figure 4.4. OPN was detected as an intense continuous band from 50 to 100 kDa in both race groups. The intensity of this band indicates that OPN is the dominant protein in CME. There is also a weak band apparent at 120 kDa which is significantly less intense compared to that at 50 – 100 kDa.

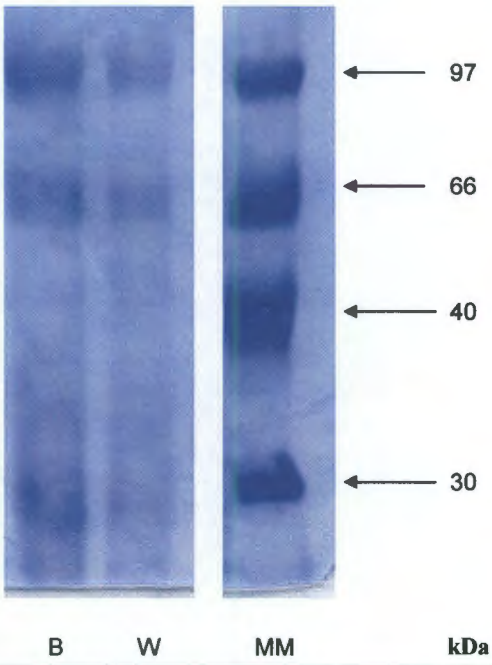


Figure 4.3: Coomassie stained 10% SDS-PAGE of CME from black and white subjects. B: Black CME, W: White CME, MM: low molecular weight marker.

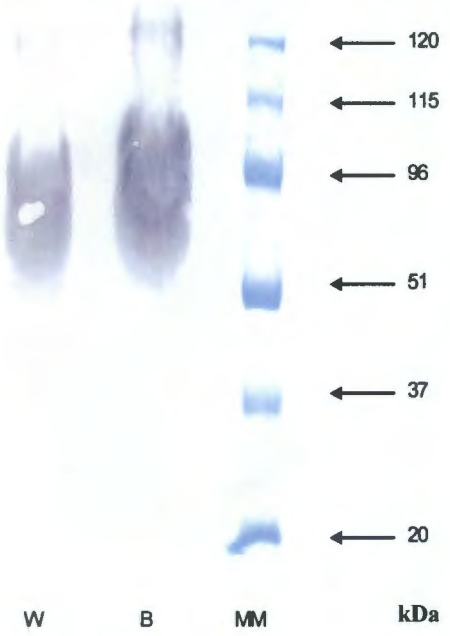


Figure 4.4: Western blot of CME from blacks and whites immunoblotted for OPN under non-reducing conditions. B: Black CME, W: White CME, MM: low molecular weight marker.

4.4.3 Bradford Assay

A Bradford assay was performed to determine the total protein concentration in BCME and WCME. Table 4.1 shows the concentrations of BSA used to plot a standard curve for the determination of the protein concentrations while the BSA standard curve is shown in Figure 4.5.

Table 4.1: Standard curve data for Bradford bovine serum albumin (BSA) protein assay.

BSA (µg/ml)	Mean	Standard deviation
1	0.081	0.024
2	0.150	0.015
3	0.198	0.006
4	0.254	0.009
5	0.316	0.020
10	0.577	0.027

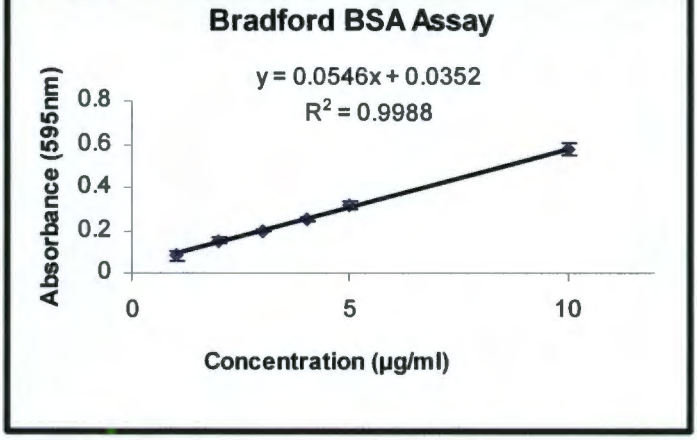


Figure 4.5: Bradford bovine serum albumin (BSA) protein assay standard curve.

Table 4.2 shows the total urinary protein concentrations from both BCME and WCME. The protein concentration ranged from 17 to 65 mg/L in black subjects and from 20 to 45 mg/L in white subjects. There is a higher mean total protein content included in BCME compared to WCME (41 *vs.* 31 mg/L) but this is not statistically significant.

Table 4.2: Total protein concentration (mg/L) from BCME and WCME.

Sample	Protein concentration (mg/L)	Sample	Protein concentration (mg/L)	BCME vs. WCME p-value
BCME1	60	WCME1	30	
BCME2	65	WCME2	30	
BCME3	55	WCME3	35	
BCME4	43	WCME4	27	
BCME5	26	WCME5	32	
BCME6	17	WCME6	45	
BCME7	20	WCME7	20	
Mean	**41 ± 5.7**	**Mean**	**31 ± 5.7**	**0.26**

4.4.4 Crystallization Experiments

Determination of CaOx MSL

The mean MSL of black and white pooled UF (n = 7) are given in Figure 4.6 (a) and (b) respectively. These show that pooled urines from black subjects have a significantly higher MSL (0.090 mol/L) than pooled urine from white subjects (0.045 mol/L), $p \leq 0.05$. Individual values are given in Appendix 2 Tables 2.1 and 2.2 and corresponding figures in Figures 2.1 and 2.2.

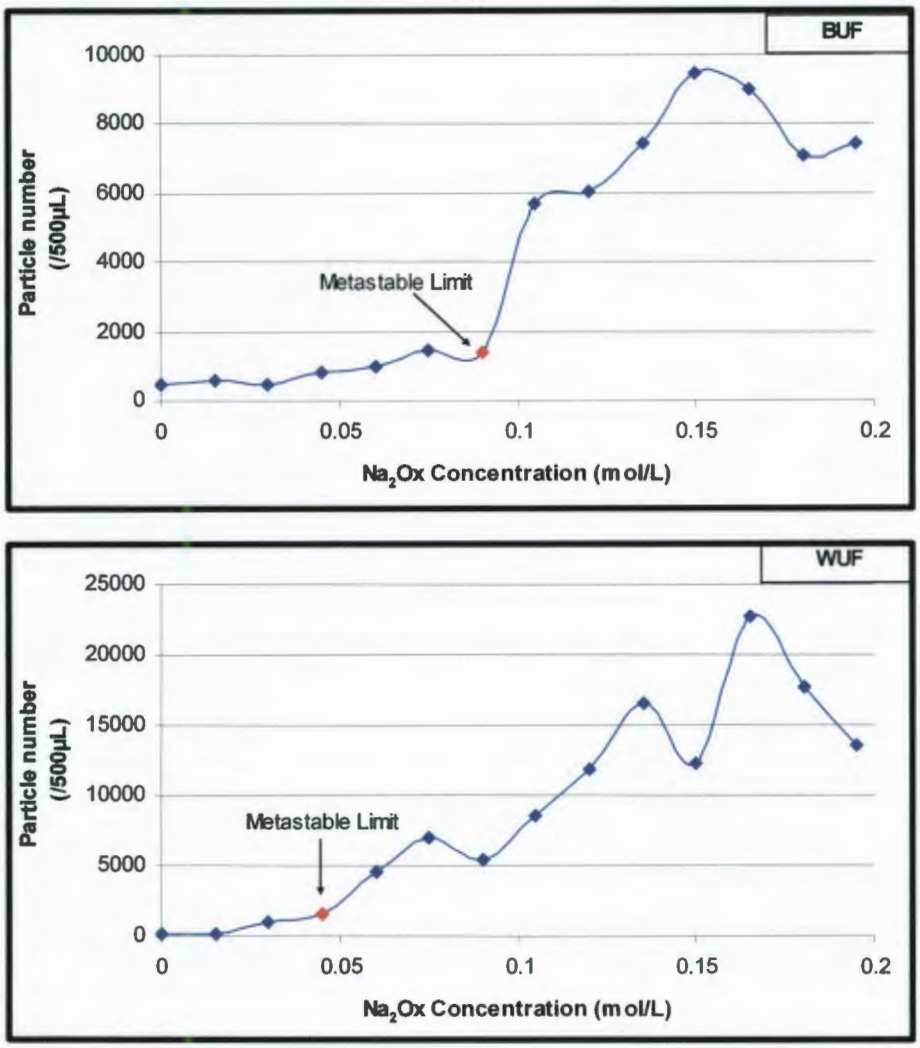


Figure 4.6: Mean MSL plots of UF from (a) black (BUF) and (b) white (WUF) subjects.

Comment

Previous work on CME protein has shown that it has no effect on the MSL (Doyle et al. 1995). This study therefore did not investigate the effect of this protein on the MSL but only compared the MSL of pooled ultrafiltered urine samples from black and white subjects. Pooled ultrafiltered samples from the former group were found to have a significantly higher MSL compared to those from the latter. This is in qualitative agreement with the results obtained for individual urines in Chapter 3. However, it is important to note that the MSLs of the ultrafiltered urines obtained for this chapter are significantly higher than those for the ultrafiltered samples in Chapter 3. The only explanation to this could be the fact that in Chapter 3 the MSL of individual samples was reported whereas in the current study it was a combined effect of 5 pooled samples.

Effect of CME on particle number

Table 4.3 shows the number of particles in BUF and WUF before and after the addition of BCME and WCME measured at 120 minutes after the addition of Na_2Ox 30 mmol/L above MSL. Figure 4.7 shows the rate of particle formation from 0 to 30 after the addition of Na_2Ox. The raw data for each experiment is presented in Appendix 2, Table 2.3 and 2.4.

In all seven BUF samples, addition of BCME or WCME induced an increase in particle number. On the other hand, while the addition of BCME to WUF again resulted in an increase in particle number, the addition of WCME caused a decrease in 5 samples.

Individual plots of the particle formation kinetics are shown in Appendix 2, Figures 2.3 and 2.4. The mean particle formation kinetics plots in Figure 4.7 show two interesting features. Firstly, the baseline rate of particle formation (i.e. in the absence of protein extract) is 100 % slower in BUF than in WUF (11.01 vs. 22.24). Secondly, addition of the extract in both ultrafiltered urines slows down the kinetics.

Table 4.3: Particle number (/500 µL) in black and white ultrafiltered urine (BUF and WUF) before and after the addition of BCME and WCME at 120 minutes, at final concentrations of 0.5 mg/L (n = 7).

Pooled Sample	BUF	BUF + BCME	BUF + WCME	p-values
1	11909	13496	12008	
2	14006	22881	24511	
3	21796	22450	25886	
4	22638	24036	23274	
5	17226	22408	19074	
6	7266	11825	8611	
7	19433	24162	20073	
Mean ± SE	**16325 ± 1533**	**20179 ± 1568**	**19062 ± 1846**	
Pooled Sample	**WUF**	**WUF + BCME**	**WUF + WCME**	**p-values**
1	20647	29755	16437	
2	19028	24288	16309	
3	21731	22264	22459	
4	22277	23914	17703	
5	20297	22929	21247	
6	19996	23082	16665	
7	18657	21641	16933	
Mean ± SE	**20376 ± 1533**	**23981 ± 1568**	**18250 ± 1846**	
Comparisons				**p-values**
BUF vs. WUF				**0.0864**
BUF + BCME vs. BUF + WCME				**0.728**
***WUF + BCME vs. WUF + WCME**				***0.001**
BUF + BCME vs. WUF + BCME				**0.112**
BUF + WCME vs. WUF + WCME				**0.763**

** indicates that the difference is statistically significant: $p \leq 0.05$*

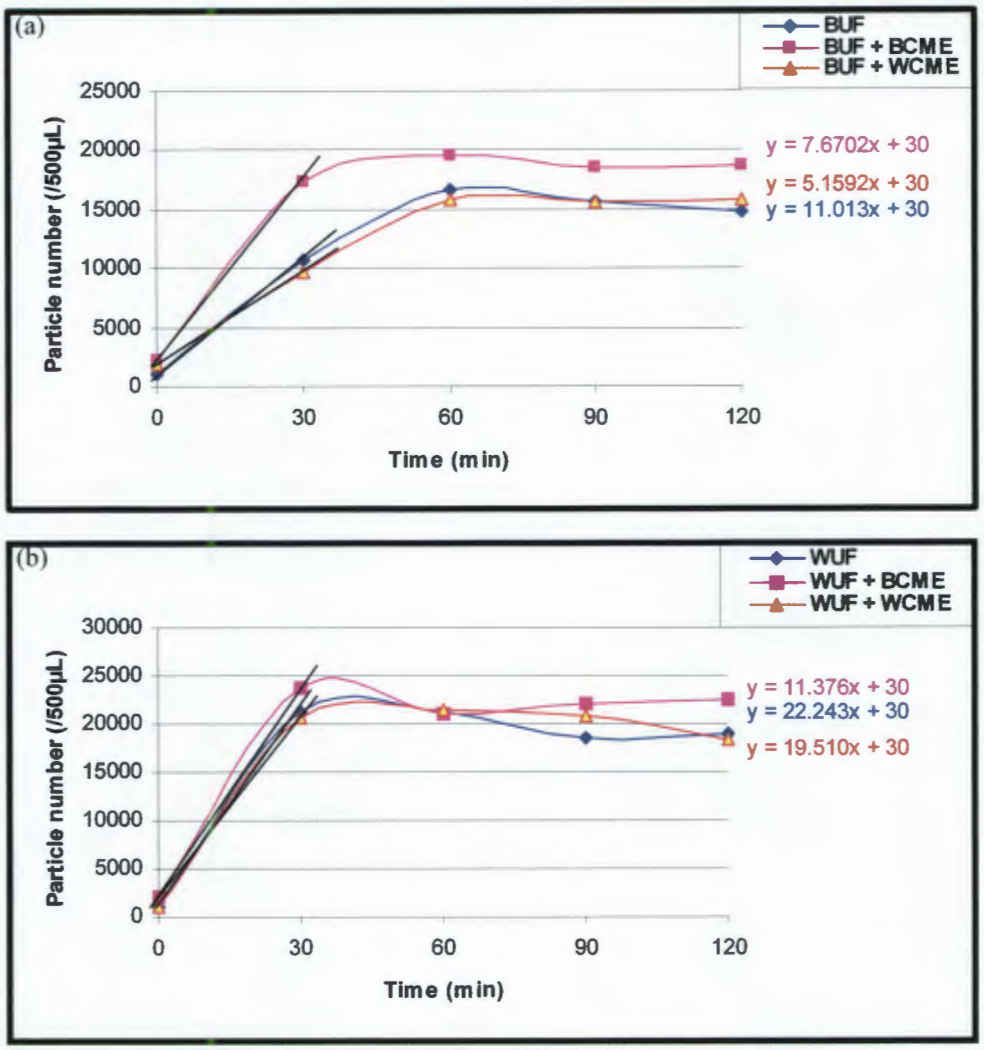


Figure 4.7: Mean plot of particle number vs. time in (a) BUF and (b) WUF before and after the addition of BCME and WCME and Na_2Ox. The rate of particle formation is given by the slope of the graph at 0 to 30 minutes (trendline) of the experiment.

Comment

These results demonstrate that BCME is a promoter of CaOx crystal nucleation and that it operates in this manner independently of the urine milieu. However, the role of WCME varies in accordance with urine composition, thereby drawing attention to the synergistic relationship between protein inhibitory performance and urine composition. Promotion of CaOx crystal nucleation kinetics is considered as a favourable phenomenon in retarding kidney stone pathogenesis as it rapidly lowers the supersaturation of urine (Hess et al. 1995). BCME has demonstrated a higher potency than WCME in this regard.

Effect of CME on particle volume

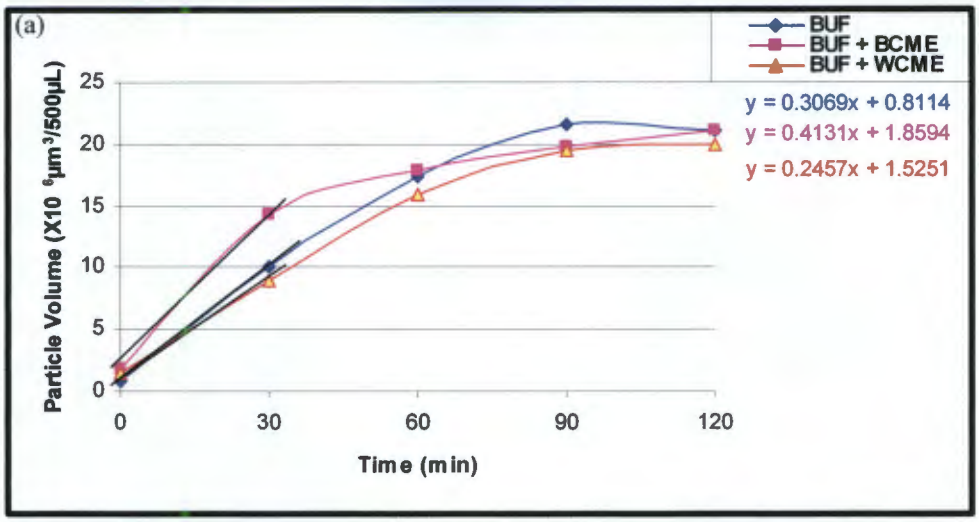
Table 4.4 and Figure 4.8 show the effect of BCME and WCME on particle volume of BUF and WUF at 120 minutes after the addition of each protein. It is readily noted that at baseline, the total particle volume is lower in BUF than in WUF. This is consistent with the previously observed lower particle number in the former. In 5 samples the addition of BCME to BUF induced an increase in particle volume whereas increases and decreases were observed for WCME. For WUF, an increase in particle volume was observed in 5 samples after the addition of both BCME and WCME. Individual values at 30 minute intervals to t = 120 minutes are in Appendix 2, Table 2.5 and 2.6.

The mean particle volume formation kinetics plots are shown in Figure 4.8. Individual plots from which the mean was plotted are shown in Appendix 2, Figures 2.5 and 2.6. The rate at which particle volume increased was much slower in BUF (range 0.25 – 0.41 μm^3/min) than in WUF (range 0.79-0.97 μm^3/min). This is consistent with the slower rate of nucleation previously reported for BUF (page 74). Of interest is that the kinetics increased when the protein extract was added to its parent urine but decreased in the cross-over experiments.

Table 4.4: Particle volume (x 10^6 μm^3/500 μL) in black and white ultrafiltered urine (BUF and WUF) before and after the addition of BCME and WCME at final concentrations of 0.5 mg/L (n = 7).

Pooled Sample	BUF	BUF + BCME	BUF + WCME	p-values
1	9.19	10.67	7.52	
2	10.72	17.76	18.96	
3	19.23	27.72	12.82	
4	36.73	29.06	27.85	
5	31.07	33.57	33.13	
6	7.00	10.86	8.14	
7	42.79	26.39	38.24	
Mean ± SE	**22.39 ± 6.565**	**22.29 ± 3.025**	**20.95 ± 5.137**	
Pooled Sample	**WUF**	**WUF + BCME**	**WUF + WCME**	**p-values**
1	19.35	20.75	29.68	
2	26.95	31.46	29.99	
3	27.01	34.38	40.83	
4	27.06	27.83	49.87	
5	30.34	33.35	39.21	
6	59.12	40.05	65.14	
7	71.96	39.02	64.21	
Mean ± SE	**37.39 ± 6.565**	**32.41 ± 3.025**	**45.56 ± 5.137**	
Comparisons				**p-values**
BUF vs. WUF				**0.132**
***BUF + BCME vs. BUF + WCME**				***0.036**
***WUF + BCME vs. WUF + WCME**				***0.005**
BUF + BCME vs. WUF + BCME				**0.822**
***BUF + WCME vs. WUF + WCME**				***0.052**

** indicates that the difference is statistically significant: $p \leq 0.05$*



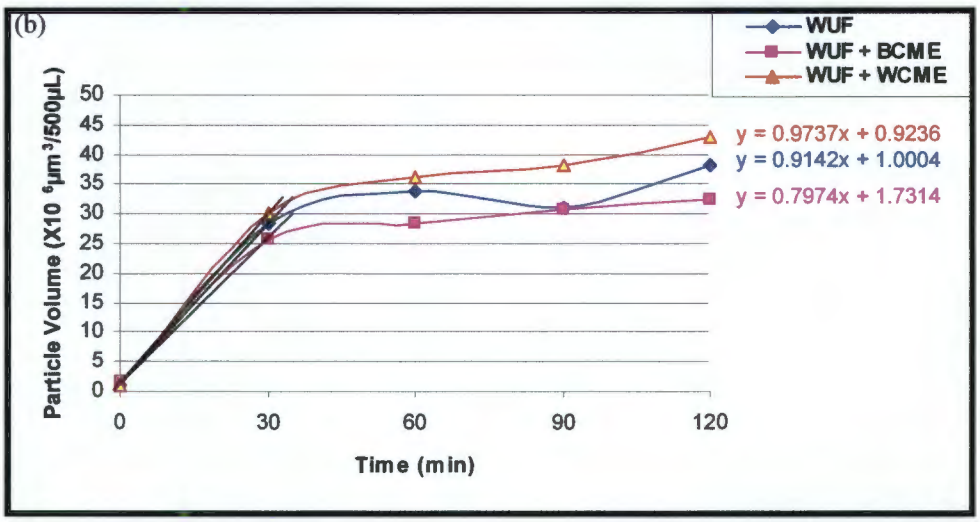


Figure 4.8: Mean plot of particle volume vs. time in (a) BUF and (b) WUF before and after the addition of BCME and WCME and Na_2Ox. The rate of particle formation is given by the slope of the graph at 0 to 30 minutes (trendline) of the experiment.

Comment

An increase in particle volume can occur as a result of several mechanisms such as;

(i) increased nucleation,

(ii) increased growth of existing particles, and

(iii) increased aggregation with concomitant entrapment of air and/or cellular debris.

It seems likely that the increases in particle volume in the present series of experiments are consistent with the previously described increases in nucleation corresponding to the addition of BCME to both BUF and WUF. The only apparent inconsistency is the observation of an increase in particle volume when WCME was added to WUF as this conflicts with the decrease in particle number reported in the previous section (page 74). However, this is readily explained by the proposition that aggregation (with entrapment of non crystalline extraneous material) is occurring. This would account for the observed decrease in particle number and the (present) increase in particle volume. The increase in kinetics when the protein extract was added to its parent urine and a decrease in the cross-over experiments draws attention to the notion that urine composition influences the ultimate behaviour of the protein extract.

Effect of CME on particle volume-size distribution

The mean particle volume-size distribution plots at 120 minutes for both BUF (n = 7) and WUF (n = 7) before and after addition of the protein are shown in Figures 4.9 (a) and (b), respectively. Individual plots are presented in Appendix 2, Figures 2.7 and 2.8.

In BUF (control), most particles lie in the size range below 15 μm, with an ill-defined peak at 3 μm. Addition of WCME has no effect on this distribution. However, addition of BCME dramatically increases the number of particles at 3 μm. As a result, there are far fewer particles in the bigger size range.

In WUF (control) the distribution is similar to that in BUF (control) except that there are more particles in all size ranges up to 50 μm. Addition of WCME reduces the number of small particles (particularly those at 3 μm), with a concomitant increase in the number of

particles in the range 10 – 30 µm. Addition of BCME dramatically increases the number of smaller particles, the consequence of which is that there are few particles bigger than 20 µm.

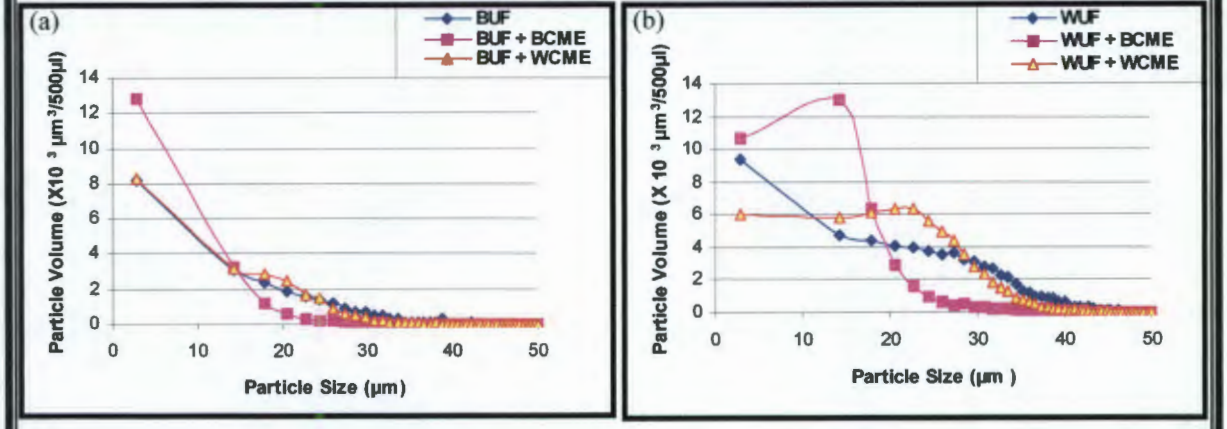


Figure 4.9: Mean particle volume-size distribution by (a) BUF and (b) WUF before and after the addition of BCME and WCME at final concentrations of 0.5 mg/L and at 120 minutes of the experiment.

Comment

Addition of BCME both in its parent urine and in WUF induced a remarkable increase in the number and a decrease in the size of particles. These results suggest that BCME is an inhibitor of growth and/or aggregation while the converse is true for WCME.

Effect of CME on [^{14}C]-oxalate deposition

The effect of CME on ^{14}C-oxalate deposition in BUF (n = 7) and WUF (n = 7) is shown in Figure 4.10 below. This is reported as the percentage of precipitated or bound oxalate at increasing time intervals from t = 0 to t = 120 minutes. Individual graphs are presented in Appendix 2, Figures 2.9 and 2.10.

It is noted that at baseline (i.e. in the absence of CME from either group), the percentage of precipitated ^{14}C-oxalate is significantly lower in BUF (41 %) than in WUF (74 %), $p < 0.05$. The graphs also show a significant decrease in the amount of deposited CaOx when CME from either group is added to BUF and that the effect is significantly greater in the presence

of BCME (42 % to 25 %) than in the presence of WCME (42 % to 32 %). Of great interest and importance is that addition of CME (from both groups) to WUF had no effect.

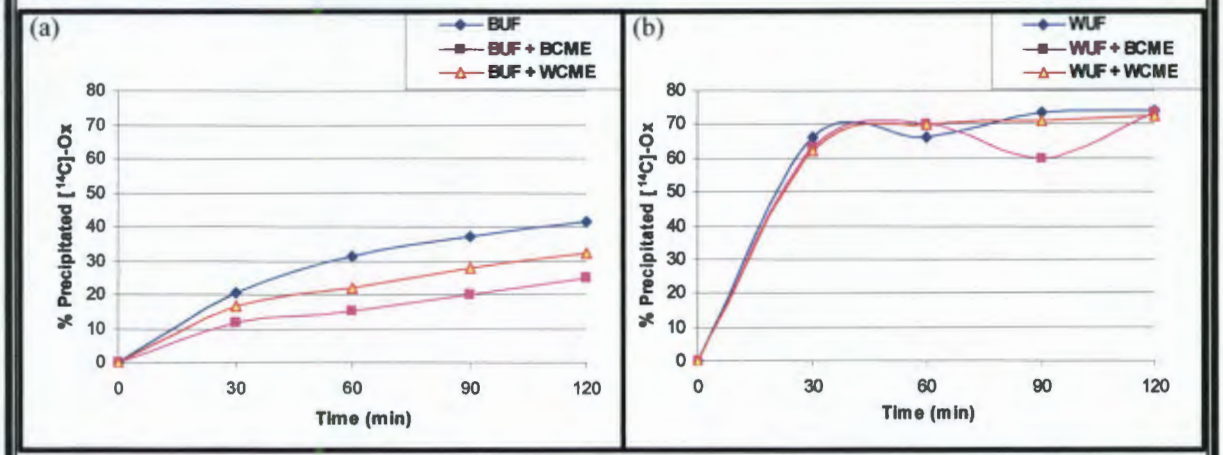


Figure 4.10: Mean percentage [^{14}C]-Oxalate deposited in (a) BUF and (b) WUF before and after the addition of BCME and WCME at final concentrations of 0.5 mg/l.

Comment

The significantly lower percentage of deposited ^{14}C-oxalate in BUF (41 %) than in WUF (74 %), $p < 0.05$ demonstrates that under exactly the same conditions, a sample of ultrafiltered urine from black subjects is more resistant to CaOx crystallization than an equivalent sample from white subjects. Addition of CME from either group to BUF retarded CaOx deposition and this was more pronounced by BCME whereas both proteins had no effect on WUF. These results show that BCME is an effective inhibitor of CaOx crystal formation in its own urine while WCME does not have the same capacity in its own urine. This is therefore an extremely important observation as it possibly contributes towards accounting for the difference in stone incidence between the black and white groups.

Effect of CME on COM and COD aggregation

(a) Effect of CME on COM crystal aggregation

The effect of CME (n = 7) on crystal aggregation was first tested in a COM crystal slurry in the absence of ultrafiltered urine. This was achieved by measuring the decline in UV absorbance as a function of time. Both BCME and WCME inhibited COM crystal aggregation (Table 4.5). BCME inhibited crystal aggregation to a significantly greater extent (91 %) compared to WCME (69 %), $p < 0.05$. A mean plot of absorption vs. time is shown in Figure 4.11. An arrow indicates the time at which stirring was stopped. Individual curves are presented in Appendix 2, Figures 2.11.

Table 4.5: Mean slopes of absorbance vs. time graphs, R^2 and percentage aggregation inhibition (I_A %) induced by BCME and WCME in COM slurry in the absence and presence of urine (n = 7).

Sample	Slope X -10^{-4} (OD/sec); (R^2)	I_A% (p-values , wrt COM)	p-values
COM	35; (0.993)		
COM + BCME	3.0; (0.989)	91 ($p < 0.05$)	
COM + WCME	11; (0.982)	69 ($p < 0.05$)	
***BCME vs. WCME**			*** 0.001**
COM + BUF	9.0; (0.997)	74 ($p < 0.05$)	
COM + BUF + BCME	3.0; (0.982)	91 ($p < 0.05$)	
COM + BUF + WCME	3.0; (0.992)	83 ($p < 0.05$)	
COM + BUF + BCME vs. COM + BUF + WCME			**0.183**
COM + WUF	6.0; (0.993)	83 ($p < 0.05$)	
COM + WUF + BCME	3.0; (0.989)	91 ($p < 0.05$)	
COM + WUF + WCME	2.0; (0.985)	94 ($p < 0.05$)	
COM + WUF + BCME vs. COM + WUF + WCME			**0.778**
Comparisons			**p-values**
BUF vs. WUF			**0.508**
COM + BUF + BCME vs. COM + WUF + BCME			**0.579**
COM + BUF + WCME vs. COM + WUF + WCME			**0.077**

** indicates that the difference is statistically significant: $p \leq 0.05$*

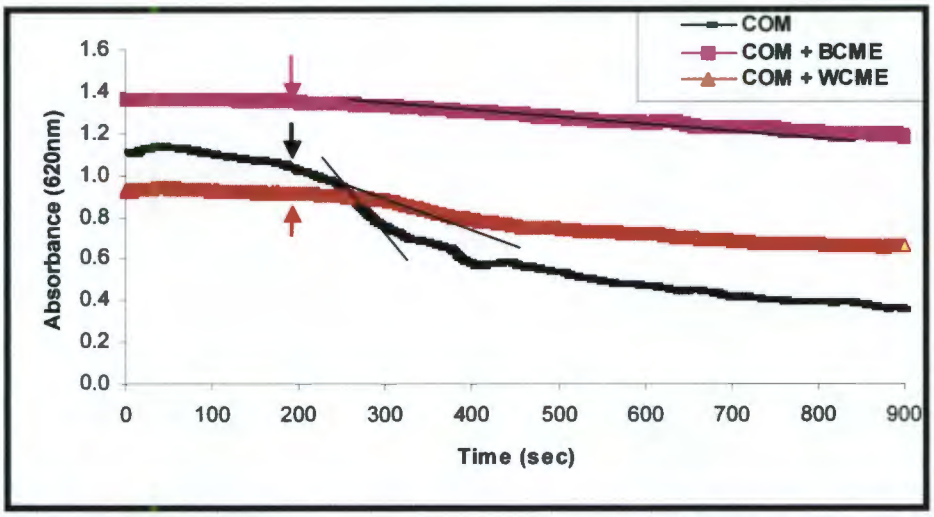


Figure 4.11: Mean COM crystal sedimentation plot of absorbance vs. time before and after the addition of BCME and WCME at final concentrations of 0.5 mg/L. An arrow indicates the time at which the stirrer was switched off and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Thereafter, the experiment was repeated in the presence of ultrafiltered urine alone (n = 7) and finally, in the presence of the combination of the latter and crystal matrix extract (n = 7). Figure 4.12 shows a mean plot of absorbance vs. time for these experiments while Table 4.5 shows the mean slopes and corresponding percentage inhibition of aggregation. Individual plots are in Appendix 2, Figures 2.12 and 2.13 and corresponding percentages of aggregation inhibition are in Appendix 2, Table 2.7.

Addition of BCME in BUF significantly increased the inhibition of COM crystal aggregation from 74 % to 91 %, the same inhibition level that BCME produced on its own without urine (Table 4.5). The addition of WCME to WUF also significantly increased the percentage inhibition, albeit by not quite the same amount (i.e. from 83 % to 94 %). Nevertheless, this was the highest level of inhibition achieved in the different experiments. In the cross-over experiments, percentage inhibition also increased relative to the control. Of the two experiments in this category, the synergistic combination of BCME in WUF was more efficacious than WCME in BUF.

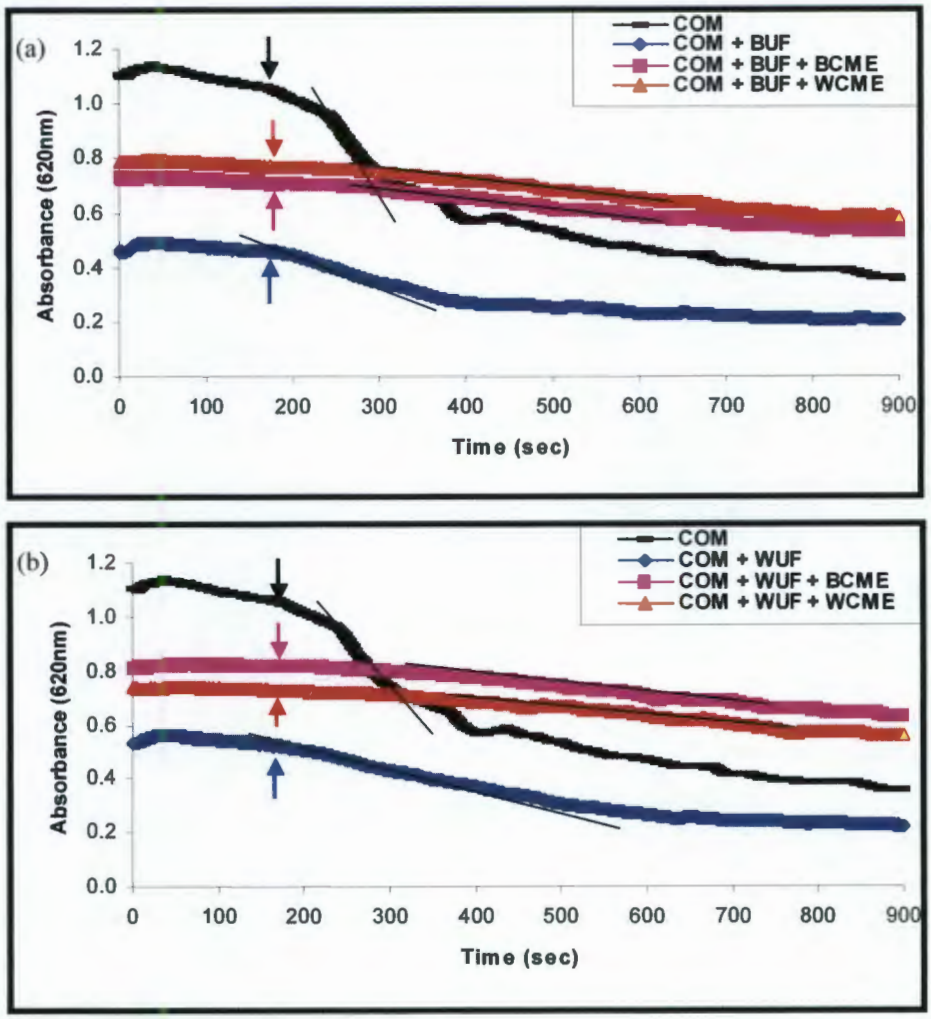


Figure 4.12: Mean COM crystal sedimentation plot of absorbance vs. time before and after the addition of BCME and WCME in (a) BUF and (b) WUF at final concentrations of 0.5 mg/L. An arrow indicates the time at which the stirrer was switched off and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Comment

It is immediately apparent that the ultrafiltered urines of both race groups have different inhibitory capacity with respect to COM crystal aggregation. In this regard WUF exerts a stronger (but statistically insignificant) effect than BUF (83 % vs. 74 %, $p > 0.05$). Interestingly, addition of CME increases the inhibition to 91 % in the black group and to 94 % in the white group. Comparison with the results obtained for the protein extract alone (Table 4.5) shows the same level of inhibition for BCME (91 %) but a lower value for WCME (69 %). In the cross-over experiments, relatively stronger inhibition was achieved by BCME in WUF (91 %) compared to WCME in BUF (83 %). While these results demonstrate that the protein extract from both groups is an inhibitor of COM crystal aggregation, they cannot differentiate between their relative strengths in this regard. Similarly, while the synergistic role of both BUF and WUF is apparent, it is not obvious which one is superior.

(b) Effect of CME on COD crystal aggregation

Table 4.6 shows percentage inhibition of aggregation of COD induced by BCME and WCME in the absence of urine. Both BCME and WCME induced approximately the same level of aggregation inhibition of COD crystals; 46 and 42 % respectively. A mean plot of absorbance vs. time (7 experiments) is shown in Figure 4.13. Individual results are given in Appendix 2, Figure 2.14, and Table 2.8.

Table 4.6 Mean slopes of absorbance vs. time graphs, R^2 and percentage aggregation inhibition (I_A %) induced by BCME and WCME in COD slurry in the absence of urine (n = 7).

Sample	Slope X -10^{-4} (OD/sec); (R^2)	I_A % (p-values , wrt COD)	p-value
COD	24; (0.991)		
COD + BCME	13; (0.983)	46 % ($p < 0.05$)	
COD + WCME	14; (0.989)	42 % ($p < 0.05$)	
BCME vs. WCME			**0.326**

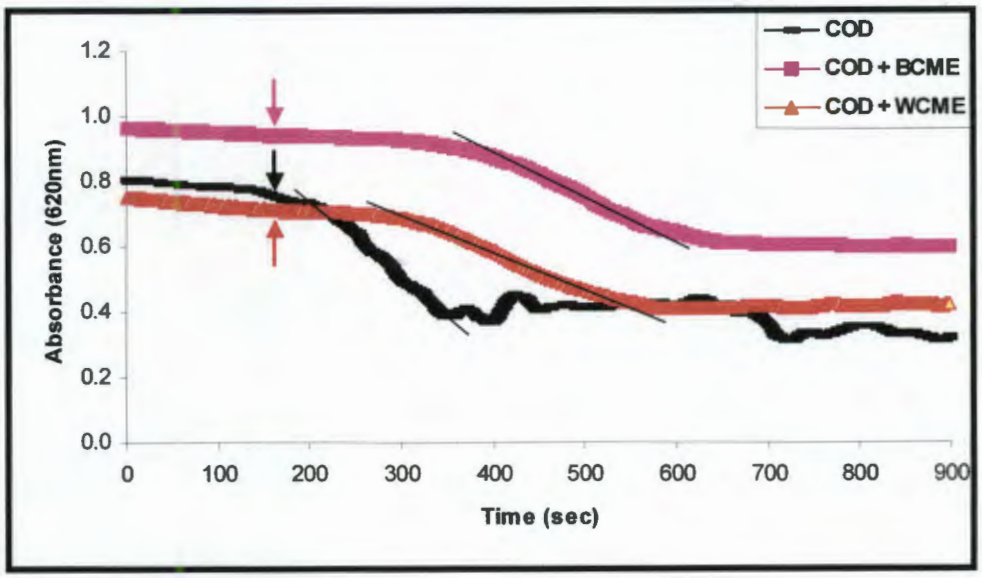


Figure 4.13: Mean COD crystal sedimentation plot of absorbance vs. time before and after the addition of BCME and WCME at final concentrations of 0.5 mg/L. An arrow indicates the time at which the stirrer was switched off and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Comment

It is immediately noted that inhibition of COD crystal aggregation by the protein extracts (46 % and 42 % for BCME and WCME, respectively) is significantly different to that of COM crystal aggregation (91 % and 69 % for BCME and WCME, respectively). The reason for this is unknown. However, it is anticipated that it is one of the defence mechanisms towards stone formation. It should be noted that inhibition of COM crystal aggregation relative to COD crystal aggregation could be of pathological importance because the former type of crystal cannot easily pass through the system as it is highly adherent to renal epithelial cells. On the other hand, COD crystals are less adherent to these cells, so even if they aggregate, to a less extent of course, they will be easily washed through.

Effect of CME on crystal nucleation

Mean values for the percentage inhibition of nucleation (n = 7) are given in Table 4.7. This shows that both BCME and WCME induced inhibition of nucleation of CaOx crystals in an inorganic solution. However, BCME (90 %) showed a significantly higher inhibition compared to WCME (64 %), $p < 0.05$. A mean plot of 7 runs is shown in Figure 4.14, individual plots are in Appendix 2, Figure 2.15 and corresponding percentages of nucleation inhibition are in Appendix 2, Table 2.9.

Table 4.7: Mean slopes of absorbance vs. time graphs, R^2 and percentage nucleation inhibition (I_N %) induced by BCME and WCME (n = 7).

Sample	Slope X -10^{-4} (OD/sec); (R^2)	I_N % (p-values , wrt control)	p-value
Control (Ctrl)	4.1; (0.991)		
Ctrl + BCME	0.4; (0.992)	90 ($p < 0.05$)	
Ctrl + WCME	1.5; (0.986)	64 ($p < 0.05$)	
***BCME vs. WCME**			*** 0.004**

** indicates that the difference is statistically significant: $p \leq 0.05$*

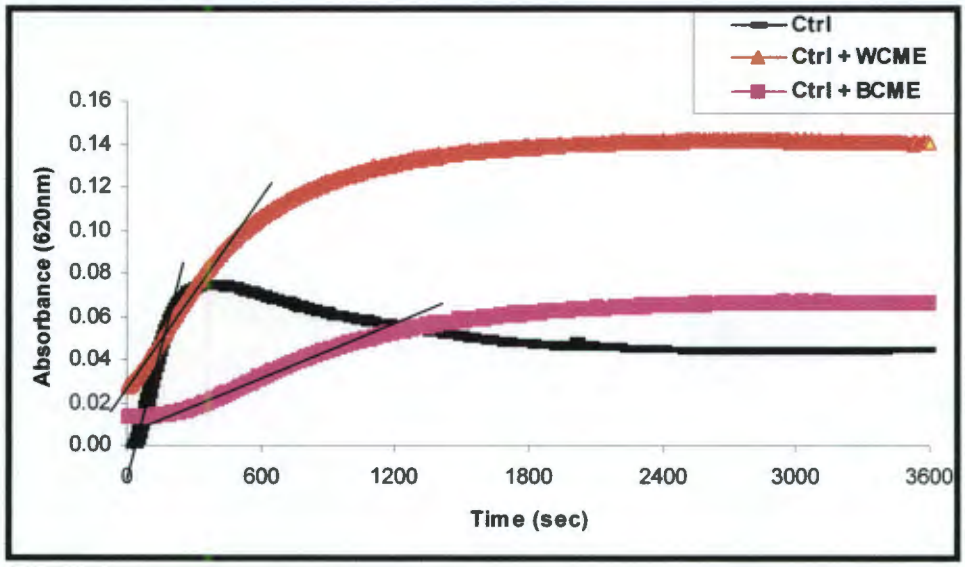


Figure 4.14: Mean CaOx crystal nucleation plots of absorbance vs. time before and after the addition of BCME and WCME at final concentrations of 0.5 mg/L. The trendline indicates the absorbance used for the calculation of nucleation inhibition.

Comment

In general, the effect of nucleation on CaOx crystallization is two-fold:

(i) It rapidly lowers urinary supersaturation without producing particles of significant sizes.

(ii) It is an essential prerequisite for further formation of larger particles within the urinary tract which might eventually form a stone.

In the context of the second point, retarding the rate of CaOx crystal nucleation might therefore be of great significance in reducing stone formation. The results reported above demonstrate that BCME (91 %) is a significant inhibitor of CaOx nucleation compared to WCME (64 %); $p = 0.004$. This observation might contribute towards understanding the rarity of stone incidence in the black population.

Effect of CME on COM crystal zeta potential

Table 4.8 shows the individual zeta potential values of the COM crystal slurry before and after the addition of BCME and WCME. Figure 4.15 shows the histogram of the mean values shown in Table 4.8. Addition of BCME to the COM slurry induced a significant increase in the zeta potential as indicated by an increase in the negative charge, whereas WCME did not change the zeta potential.

Table 4.8: Zeta potential (mV) of COM crystals before and after addition of BCME and WCME (n = 9).

Number of runs	COM	COM + BCME	COM + WCME	BCME vs WCME p- value
1	-10.3	-15.8	-11.8	
2	-12.8	-18.9	-13.4	
3	-15.3	-20.8	-15.8	
4	-7.80	-15.4	-5.76	
5	-10.8	-14.1	-11.6	
6	-11.2	-16.0	-13.2	
7	-12.5	-16.3	-13.6	
8	-14.3	-18.2	-16.2	
9	-14.8	-9.81	-12.9	
Mean ± SE	**-12.2 ± 0.92**	**-16.2 ± 0.92 (0.01)**	**-12.7 ± 0.92 (0.419)**	***0.031**

** indicates that the difference is statistically significant: $p \leq 0.05$*

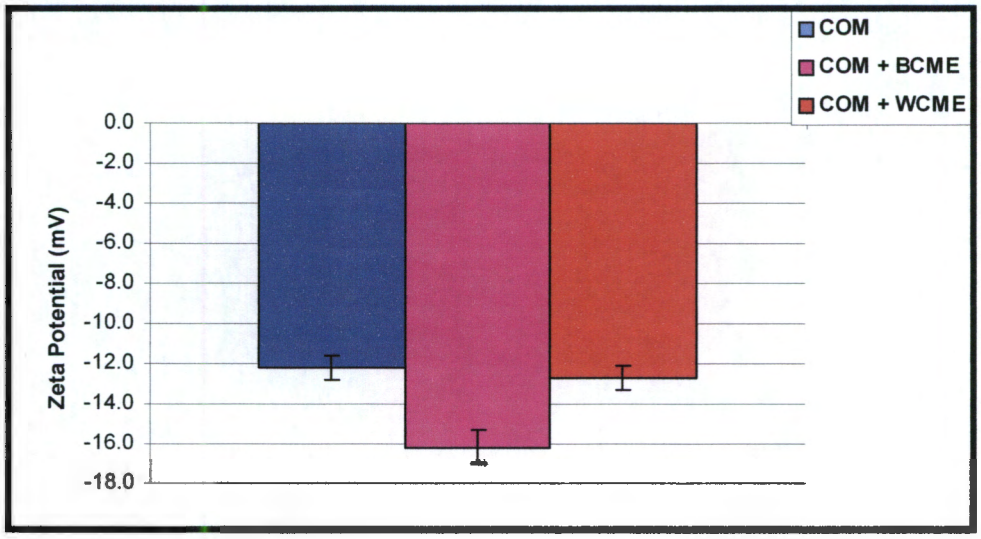


Figure 4.15: Mean zeta potential histogram before and after the addition of BCME and WCME to COM crystal slurry.

Effect of CME on COD crystal zeta potential

Table 4.9 shows the individual zeta potential values of the COD crystal slurry before and after the addition of BCME and WCME. Figure 4.16 shows the histogram of the mean values shown in Table 4.9. Addition of BCME to the COD slurry induced a significant increase in the zeta potential as indicated by an increase in the negative charge, whereas WCME induced a decrease in the zeta potential. Comparing BCME and WCME, the negative charge induced by BCME is significantly higher than that resulting from the latter.

Table 4.9: Zeta potential of COD crystals before and after the addition of BCME and WCME (n = 9).

Number of runs	COD	COD + BCME	COD + WCME	BCME vs. WCME p- value
1	-12.1	-16.6	-11.0	
2	-14.9	-17.2	-10.7	
3	-15.8	-19.6	-9.73	
4	-10.5	-17.4	-12.5	
5	-11.1	-18.4	-16.7	
6	-9.27	-17.0	-11.6	
7	-10.6	-12.8	-14.2	
8	-12.1	-14.2	-12.2	
9	-12.2	-12.2	-10.9	
Mean ± SE	**-12.1 ± 0.77**	**-16.2 ± 0.775 (0.01)**	**-9.79 ± 1.93 (0.419)**	***0.036**

** indicates that the difference is statistically significant: $p \leq 0.05$*

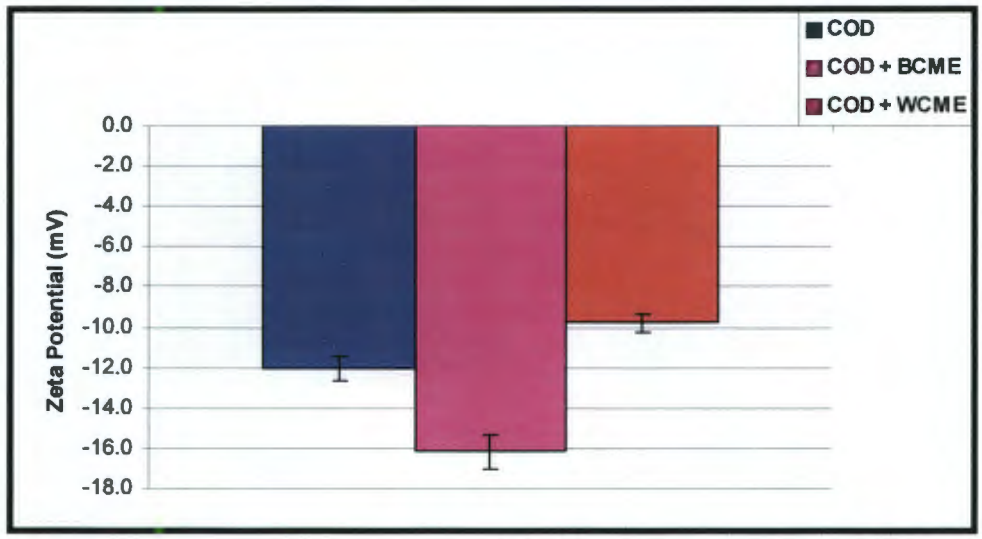


Figure 4.16: Mean zeta potential histogram before and after the addition of BCME and WCME to COD crystal slurry.

Comment

These results demonstrate that BCME increases the magnitude of the negative zeta potential in COM crystals from -12.2 to -16.2 ($p < 0.05$), and in COD crystals from -12.1 to -16.2 ($p < 0.05$) but that WCME has no such effect. Since a shift to more negative zeta potential is indicative of a greater capacity to inhibit crystal aggregation, it is concluded that BCME operates in this manner while WCME does not.

4.5 DISCUSSION

The OPN immunoblot reported in this chapter (Figure 4.5) demonstrated that OPN is indeed the major protein included in COD crystals. This observation is in agreement with the results reported by several authors (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005). OPN appeared as a continuous band from 50 to 100 kDa in both race groups. This might be due to degradation as OPN has been reported to be easily degraded by urinary proteases and no protease inhibitors were added to the samples (Bautista et al. 1996; Ryall et al. 2005). The band appearing at 120 kDa in both lanes may be aggregates of OPN (Johnson-Tardieu et al. 2000, Webber et al. 2003).

One of the main objectives of this chapter was to test the inhibitory effect of COD-CME towards CaOx crystallization using various crystallization techniques. As explained earlier, the effect of nucleation on CaOx crystallization can be two-fold. Paradoxically, both promotion and inhibition of CaOx crystal nucleation are considered beneficial in this context. The results obtained for particle number and volume demonstrate that BCME is a promoter of CaOx crystal nucleation and this is independent of the urine milieu whereas for WCME this varies according to the urine composition. This observation in WCME indicates a synergistic role that exists between the urine milieu and the protein.

Promotion of crystal nucleation is considered as a favourable process since it lowers urinary supersaturation without producing particles of significant sizes thus reducing the risk of forming stones (Hess et al. 1995). On the other hand, the actual nucleation assay demonstrated a highly significant inhibition of CaOx crystal nucleation by BCME (91 %) compared to WCME (64 %); $p = 0.004$. Nucleation is an essential prerequisite for further formation of larger particles within the urinary tract which might eventually form a stone (Hess et al. 1995). Therefore, retarding the rate of nucleation might therefore be of great pathological significance in order to reduce the rate of particle formation. The results reported here are contradictory in that they demonstrate that BCME is both a significant inhibitor and promoter of CaOx nucleation. It is therefore not possible to draw any conclusions from these particular experiments

Particle size is also of great pathological importance as it determines whether particles will be trapped in the urinary tract or not (Hess and Kok 1996). A decrease in particle size

after the addition of CME is an indication of the role of this protein in delaying and reducing growth of CaOx crystals. The high reduction in size following addition of BCME compared to WCME suggests that the former is an inhibitor of CaOx crystal growth and aggregation. Unfortunately, SEM studies for this experiment were not performed.

Because of the reported limitations of the Coulter Counter in distinguishing between a single particle and an aggregate (Ryall et al. 1995), ^{14}C-oxalate deposition experiments were conducted in this study to supplement the Coulter counting and sizing data. The significantly lower CaOx deposition observed in BUF suggests high inhibition of CaOx formation by the black group. A more pronounced retardation of CaOx formation by BCME relative to WCME in BUF shows that the former is an effective inhibitor of CaOx crystal formation. However, neither BCME nor WCME had an effect in WUF. This is therefore yet another important observation for possibly accounting for the difference in stone incidence between the black and white groups.

The higher percentage of inhibition of both COM and COD crystal aggregation by BCME compared to WCME is another indication of the potency of the former in reducing the risk of forming stones. This observation has been supported by the zeta potential results which determine the capacity of a protein to disaggregate the crystals. As stated earlier, the more negative the zeta potential, the higher the inhibition of crystal aggregation. BCME resulted in a more negative charge of COM and COD crystals (from -12.1 to -16.2 mV for both crystal phases) whereas WCME induced either a decrease in zeta potential or no change at all (-12.1 to -9.97 mV and -12.2 to -12.7 mV for COD and COM crystals, respectively). The increase in the negative charge arising from BCME might be due to the acidity of the proteins (Finlayson et al. 1984) in CME. Interestingly and most importantly, OPN is the most abundant and highly acidic protein in the latter (Kohri et al. 1990). The decrease in the zeta potential induced by WCME might be an indication of promotion of aggregation by this protein whereas BCME has demonstrated a strong capability to disaggregate both COM and COD crystals.

Since OPN is the main intracrystalline protein in COD, it is proposed that it may provide superior protection against CaOx crystallization in the urine of black subjects compared to whites, thereby contributing to the stone rarity in the former. However, the contribution of UPTF1 towards crystallization inhibition, if any, would be of great interest.

CHAPTER FIVE

INHIBITORY CAPACITY OF COMMERCIAL OSTEOPONTIN (OPN)

5.1 INTRODUCTION

The process of kidney stone formation is inhibited by substances in urine that reduce the concentrations of calcium and oxalate that are required for new crystal formation, growth and aggregation (Coe et al. 1992). These substances also block the adhesion of calcium oxalate monohydrate (COM) crystals to renal epithelial cells (Lieske and Toback 1993). It has been reported that the majority of inhibition of crystal growth and aggregation observed in normal urine is due to the presence of macromolecules rather than lower molecular weight molecules (Nakagawa et al. 1983; Ryall 1997; Lieske et al. 1995). About 70-90 % of these macromolecules are glycoproteins (Médétognon-Benissan et al. 1999). Most of these proteins are rich in aspartic and glutamic acid which facilitate their binding to calcium ions. They contain an Arg-Gly-Asp (RGD) cell binding sequence that allows them to adhere to cells and in turn inhibit urinary tract stone formation (Kohri et al. 1990). Osteopontin (OPN) is one of these glycoproteins which have an RGD sequence and is also rich in aspartic and glutamic acid (Kohri et al. 1990; Chen et al. 1992). It has been reported to be one of the major components of the organic matrix of stones (Hoyer 1994) and probably the source of the high percentage of acidic amino acids found in stones (Kohri et al. 1990).

As stated in Chapter 1, most researchers have reported that OPN inhibits nucleation, growth and aggregation of CaOx crystals (Prince et al. 1960; Shiraga et al. 1992; Denhartt and Guo 1993; Asplin et al. 1998; Hoyer 1994; Min et al. 1998) as well as attachment of crystals to renal epithelial cells (Shiraga et al. 1992; Chalko et al. 1992; Worcester et al. 1995; Lieske et al. 1999; Konya et al. 2003; Wesson et al. 2003) at concentrations which are lower than those in urine (1.9 μg/mL) (Lieske et al. 1995). This is mainly attributed to its high amino acid content and its ability to convert COM crystals to COD crystals which are less adherent to renal tubular cells.

Chapter 4 addressed the role of calcium oxalate dihydrate (COD) crystal matrix extract (CME) in the inhibition of CaOx crystallization. OPN was detected as the main intracrystalline protein in COD-CME. Notwithstanding the fact that there might be other proteins in COD-CME, the inhibition of CaOx crystallization by COD-CME was mainly attributed to OPN as it has been previously reported that it is the main intracrystalline protein in COD (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005). This notion was also supported by the gel and OPN immunoblot reported in Chapter 4, page 70.

Moreover, the inhibitory capacity of COD-CMEs from the black and white race groups was found to be dependent on the composition of the urine in which they were tested. It is this latter result that provided the motivation and rationale for undertaking an investigation of the behaviour of commercial OPN in inorganic solutions as well as in ultrafiltered urine from black and white subjects.

5.2 OBJECTIVES

- Test the relative inhibitory capacity of commercial OPN in urine of black and white healthy South African male subjects by conducting several crystallization experiments.
- Test the effect of commercial OPN on the zeta potential of COM and COD crystals.

5.3 Materials and Methods

5.3.1 Urine collection and treatment

Five pools of five 24 hour urine samples per pool (500 mL of each sample were used) from 25 different black (B) and 25 different white (W) healthy males were obtained without preservatives and treated separately. The samples were tested and treated as previously described in Chapter 2. After filtration (0.75 μm and 0.45 μm) the samples were ultrafiltered through a 10 kDa cut-off membrane (Millipore Corporation; Bedford USA). The ultrafiltrate was used for all the crystallization experiments.

5.3.2 Crystallization Experiments

CaOx Metastable Limit

The metastable limit of the ultrafiltrate was determined as explained (and results reported) in Chapter 2 following the method described by Ryall et al. (1985). The metastable limit was not determined in the presence of the protein because previous reports have shown that this physicochemical parameter is not affected by OPN (Doyle et al. 1995; Grover et al. 1998).

Effect of OPN on particle number, volume and size

This experiment was performed as described in Chapter 2. Briefly, two conical flasks each containing 50 mL of urine were set up. The first flask contained UF only (control). The second flask contained both UF and OPN at a final concentration of 0.5 mg/L. The OPN used was a recombinant human protein supplied by R & D Systems, Inc., USA. After the addition of 10 % (v/v) of Na_2Ox at a concentration of 30 mmol/L above the previously determined MSL, the zero time particle number, volume and size distribution were determined using a Coulter Multisizer II (Coulter Electronics Ltd, England) before the addition of OPN. The same measurements were repeated at 30 minute intervals during a 120-minute incubation period (37 °C, 100 rpm) after the addition of OPN.

Scanning Electron Microscopy

Crystals were prepared for scanning electron microscopy as previously described in Chapter 2 and were viewed under the same operating conditions except that the probe current was changed (50 pA).

Effect of OPN on [^{14}C]-oxalate deposition

This experiment was conducted as previously described in Chapter 2. Two soda-lime glass bottles each containing 30 mL of urine. The first one contained UF alone (control) and the second one with UF and OPN at a final concentration of 0.5 mg/L of OPN. The experiment was continued as previously described in Chapter 2 and the percentage of precipitated ^{14}C-oxalate induced by OPN was determined.

Effect of OPN on COM and COD crystal aggregation

This experiment was conducted according to the method described in Chapters 2 and 4. As in Chapter 4, the inhibition of COM crystal aggregation was determined in the presence of UF alone, OPN alone and the combination thereof. The protocol was repeated using COD crystals except that the inhibition was determined in the absence of UF.

Effect of OPN on CaOx crystal nucleation

The effect of OPN on CaOx crystal nucleation was determined as previously described in Chapters 2 and 4. The inhibition of nucleation was tested in the presence of urine or protein alone or a combination of both.

Effect of OPN on COM and COD crystal Zeta Potential

The effect of OPN on the zeta potential was determined as previously described in Chapter 2 at a final concentration of 0.5 mg/L.

5.4 RESULTS

5.4.1 Urine composition and physicochemical properties of pooled 24 hour samples

The mean composition and physicochemical parameters of pooled 24 hour urine samples from black and white subjects are shown in Table 5.1 (n = 5). Individual parameters for each sample from both groups are shown in Appendix 3, Tables 3.1 and 3.2. Phosphate, potassium, relative supersaturation (RS) of brushite and RS of uric acid showed a significant difference ($p \leq 0.05$) between the two groups and are denoted by * next to the p-value.

Table 5.1: Mean composition and physicochemical parameters (± SE) of pooled 24 hour urine samples (n = 5) from black and white male subjects.

Parameters	B	W	p-value
pH	5.75 ± 0.23	5.99 ± 0.23	0.230
Volume (mL/24hr)	3970 ± 658	4722 ± 658	0.441
Calcium (mmol/24hr)	9.87 ± 1.12	10.9 ± 1.12	0.508
Citrate (mmol/24hr)	13.3 ± 2.91	12.9 ± 2.91	0.934
Chloride (mmol/24hr)	519 ± 72.4	505 ± 72.4	0.890
Creatinine (mmol/24hr)	47.4 ± 6.59	58.4 ± 6.59	0.273
Magnesium (mmol/24hr)	9.23 ± 1.93	10.7 ± 1.93	0.624
Oxalate (mmol/24hr)	1.17 ± 0.240	0.800 ± 0.240	0.305
Phosphate mmol/24hr)	59.6 ± 10.9	94.6 ± 10.9	*0.051
Potassium (mmol/24hr)	131 ± 42.3	279 ± 42.3	*0.043
Sodium (mmol/24hr)	464 ± 133	391 ± 133	0.719
Sulphate (mmol/24hr)	62.3 ± 11.4	71.7 ± 11.4	0.582
Uric acid (mmol/24hr)	9.88 ± 1.69	12.1 ± 1.69	0.397
RS Brushite	0.291 ± 0.022	0.614 ± 0.022	*0.022
RS CaOx	4.96 ± 0.453	3.29 ± 0.453	*0.031
RS Uric acid	3.23 ± 0.323	1.98 ± 0.323	0.321

**Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

5.4.2 Crystallization Experiments

CaOx Metastable Limit

Figure 5.1 below shows the mean MSL plots of pooled (a) BUF and (b) WUF respectively (n = 5). There is no difference observed in the mean plots between BUF and WUF as they both have 0.75 mol/L as the MSL. The individual metastable limit plots and tables from which these means have been calculated are shown in Appendix 3, Tables 3.3 and 3.4 and Figures 3.1 and 3.2.

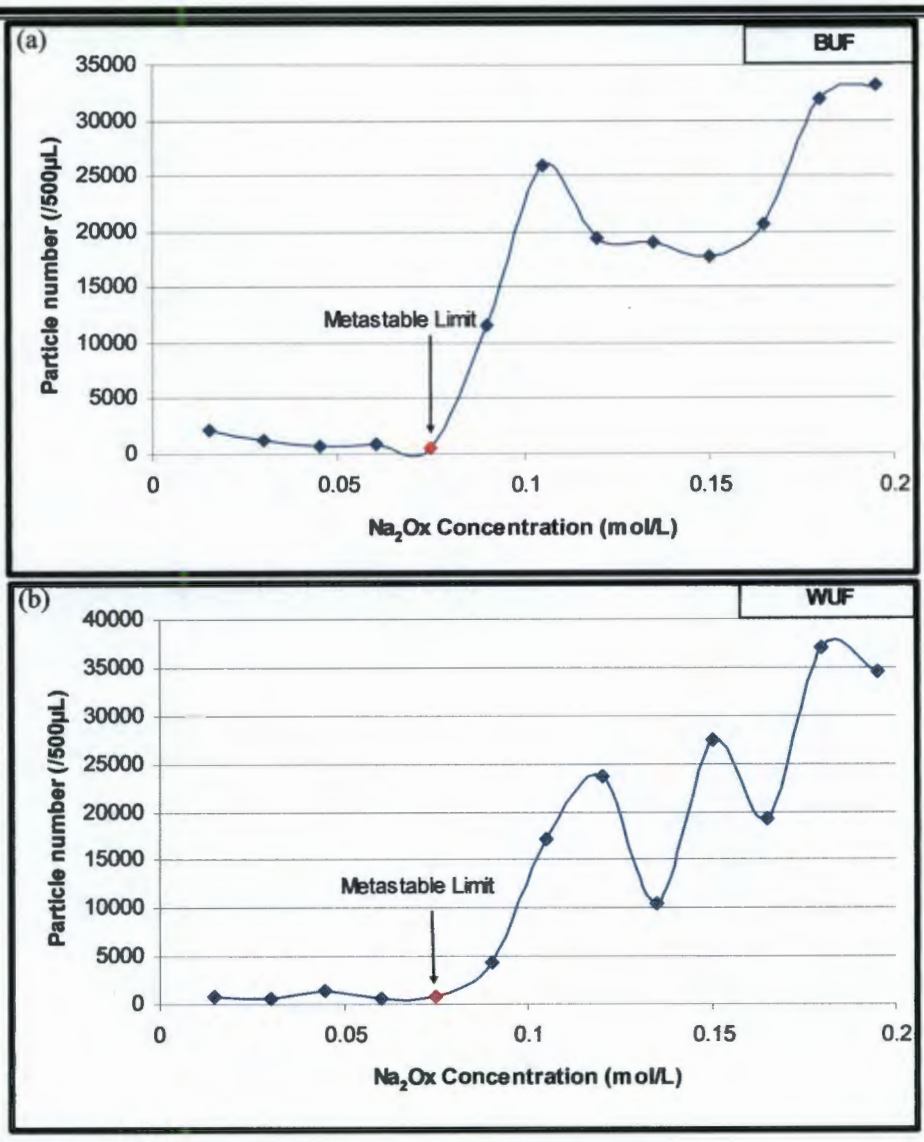


Figure 5.1: Mean MSL plots of UF from (a) black (BUF) and (b) white (WUF) subjects.

Comment

The observation of identical MSL values for BUF and WUF contradicts the finding of a higher value for BUF reported in Chapter 4. This contradiction can be attributed to uncontrollable compositional effects arising during the pooling process.

Effect of OPN on particle number

Table 5.2 shows the particle number of individual BUF (n = 5) and WUF (n = 5) samples before and after the addition of OPN. Figure 5.2 shows the rate of particle formation from 0 to 30 minutes after the addition of Na_2Ox. Individual values at 30-minutes intervals of the experiment are shown in Appendix 3, Tables 3.5 and 3.6. Addition of commercial OPN to BUF induced a decrease in particle number in four of the five urine samples from black subjects. The same trend was observed in all five of the WUF samples after the addition of commercial OPN. The mean number of particles was approximately the same for BUF and WUF before and after the addition of OPN.

It is also seen that the rate of particle formation was slower in WUF than in BUF while addition of OPN slowed the rate in both urine samples (Figure 5.2). Individual plots are shown in Appendix 3, Figures 3.3 and 3.4.

Table 5.2: **Particle number (/500 µL) in ultrafiltered pooled urine from black (BUF, n = 5) and white (WUF, n =5) subjects before and after the addition of commercial OPN at 120 minutes of the experiment at a final concentration of 0.5 mg/L.**

Pooled Sample	BUF	BUF + OPN	p-values
1	32144	39443	
2	38436	19782	
3	23771	22044	
4	16031	5679	
5	12349	11120	
Mean ± SE	**24546 ± 4693**	**19613 ± 5803**	
Pooled Sample	**WUF**	**WUF + OPN**	
1	25175	5520	
2	21644	20995	
3	13171	8448	
4	40735	37754	
5	28677	24850	
Mean ± SE	**25880 ± 4693**	**19513 ± 5803**	
Comparisons			**p-values**
BUF vs. WUF			**0.846**
BUF vs. BUF + OPN			**0.532**
WUF vs. WUF + OPN			**0.414**
BUF + OPN vs. WUF + OPN			**0.991**

**** indicates that the difference is statistically significant: $p \leq 0.05$***

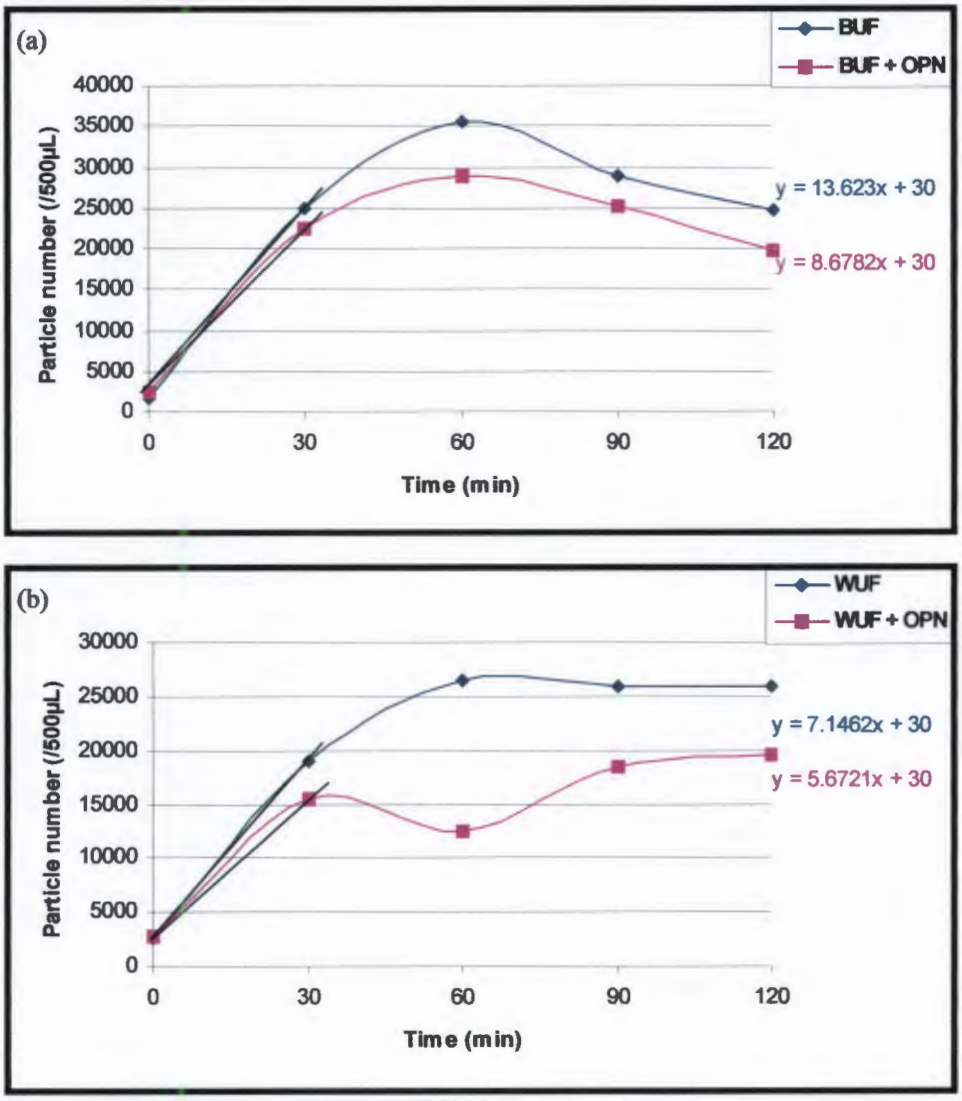


Figure 5.2: Mean plot of particle number vs. time in (a) BUF and (b) WUF before and after the addition of OPN and Na_2Ox. The rate of particle formation is given by the slope of the graph at 0 to 30 minutes of the experiment.

Comment

If it is assumed that the particles which are being measured are indeed CaOx crystals, the results for this study suggest than OPN is an inhibitor of nucleation. No distinction is possible between the synergistic roles (if any) between BUF and WUF. Crystallization experiments, besides those conducted using the Coulter Counter, will allow a conclusion to be reached concerning the true nature of the "particles".

Effect of OPN on particle volume

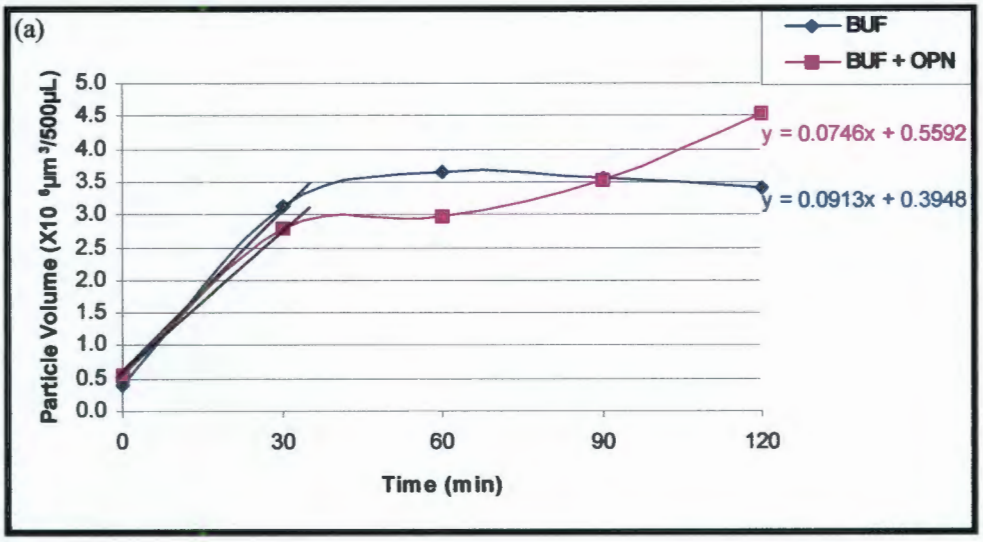
Table 5.3 shows particle volume of individual BUF (n = 5) and WUF (n = 5) samples before and after the addition of OPN. Figure 5.3 shows the rate of particle formation from 0 to 30 minutes after the addition of Na_2Ox. Individual values at 30-minute time intervals are shown in Appendix 3, Tables 3.7 and 3.8. OPN resulted in a decrease in particle volume in three out of five BUF samples, while an increase was observed in two samples. The particle volume in WUF decreased after the addition of OPN in four out of five samples, while an increase was observed in only one sample.

Addition of OPN to both BUF and WUF induced a decrease in the rate of particle volume as shown by the decrease in the slope of the graph from 0.091 to 0.076 μm^3/min and 0.118 to 0.072 μm^3/min, respectively (Figure 5.2). Individual plots are in Appendix 3, Figures 3.5 and 3.6.

Table 5.3: **Particle volume (x 10^6 μm^3/500 µL) in ultrafiltered pooled urine from black (BUF, n = 5) and white (WUF, n = 5) subjects before and after the addition of commercial OPN at 120 minutes at a final concentration of 0.5 mg/L.**

Pooled Sample	BUF	BUF + OPN	p- values
1	4.231	8.753	
2	4.196	2.168	
3	2.229	1.979	
4	4.254	1.111	
5	2.115	8.737	
Mean ± SE	**3.405 ± 0.481 (0.246)**	**4.549 ± 1.234 (0.990)**	
Pooled Sample	**WUF**	**WUF + OPN**	
1	3.876	1.344	
2	1.435	1.669	
3	2.957	1.101	
4	1.601	0.997	
5	2.896	2.567	
Mean ± SE	**2.553 ± 0.481 (0.246)**	**1.536 ± 1.234 (0.990)**	
Comparisons			**p-values**
BUF vs. WUF			**0.246**
BUF vs. BUF + OPN			**0.541**
WUF vs. WUF + OPN			**0.092**
***BUF + OPN vs. WUF + OPN**			***0.012**

**** indicates that the difference is statistically significant: $p \leq 0.05$***



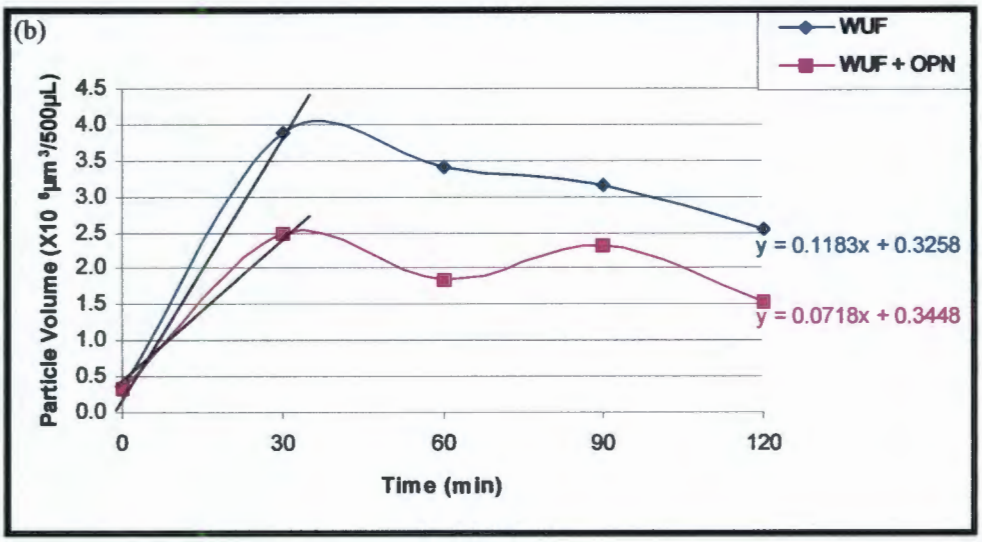


Figure 5.3: Mean plot of particle volume vs. time in (a) BUF and (b) WUF before and after the addition of OPN and Na_2Ox. The rate of particle formation is given by the slope of the graph at 0 to 30 minutes of the experiment.

Comment

The reduction in particle volume is consistent with the notion (obtained from particle numbers) that OPN is an inhibitor of CaOx crystal nucleation. Results for WUF were more convincing in this regard.

Effect of OPN on particle volume-size distribution

OPN induced a reduction in particle size in both BUF (n = 5) and WUF (n = 5). In BUF, the average size decreased from 5.83 to 4.74 μm (19 % reduction) compared to a 10 % reduction induced by OPN in WUF (3.64 to 3.28 μm). The mean particle volume-size distribution plots at 120 minutes for both BUF and WUF are presented in Figures 5.4 (a) and (b), respectively. Individual plots are indicated in Appendix 3, Figures 3.7 and 3.8.

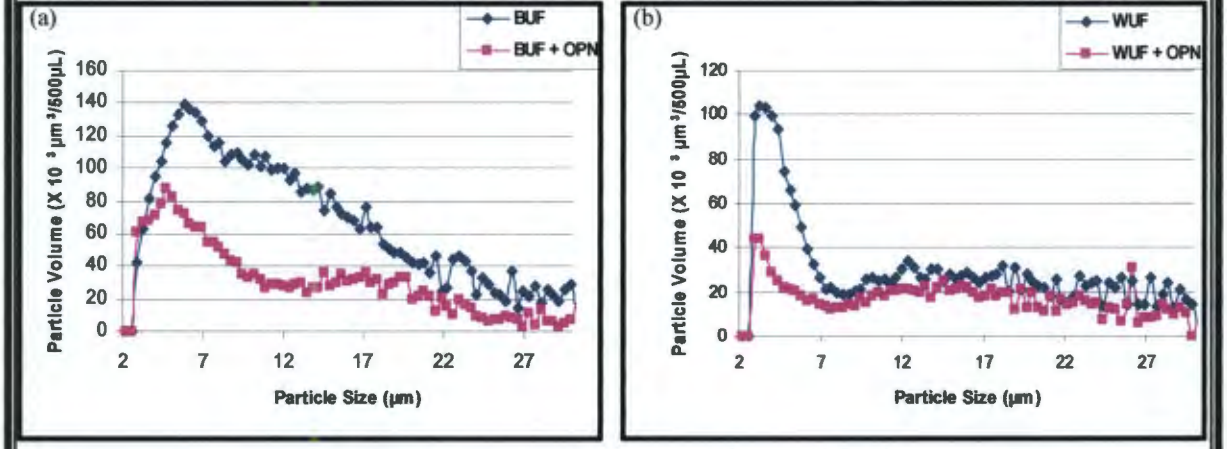


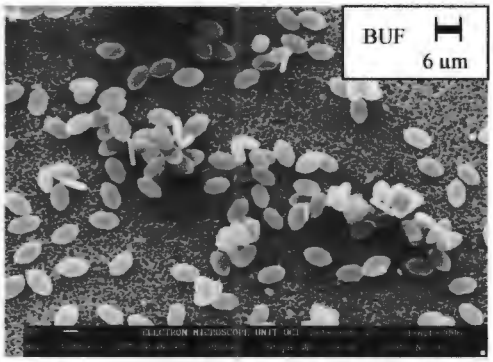
Figure 5.4: Mean particle volume-size distribution of (a) BUF and (b) WUF before and after the addition of OPN at a final concentration of 0.5 mg/l at 120 minutes of the experiment.

Comment

These results suggest that OPN is an inhibitor of CaOx crystal growth or aggregation or both. As with the other interpretations of Coulter data, the additional crystallization experiments which are described hereafter will help to reach firmer conclusion.

Scanning Electron Microscopy (SEM)

Representative scanning electron micrographs of crystals deposited after the induction of CaOx crystallization in ultrafiltered urine samples of black and white subjects before and after the addition of OPN are shown in Figures 5.5 and 5.6, respectively. Small loose and clustered COM crystals (6 μm) were deposited in BUF (Figure 5.5, left panel). Addition of OPN to BUF appeared to reduce the number and size of COM crystals (from 6 μm to 3 μm) but did not change the morphology of CaOx crystals deposited (Figure 5.5, right panel). A mixture of small COM (3 μm) and COD crystals (6 μm) were deposited in WUF (Figure 5.6, left panel). The addition of OPN did not change the number and size of these crystals (Figure 5.6, right panel). Instead an aggregate of COM crystals clustered around COD crystals was formed (Figure 5.6, right panel).



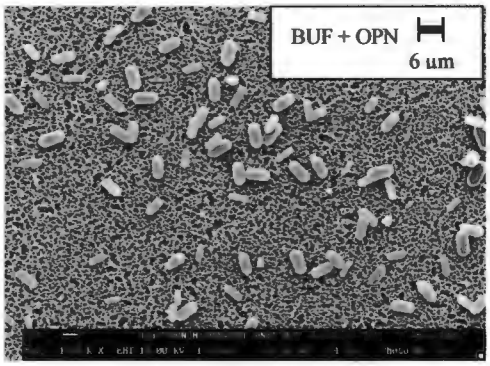
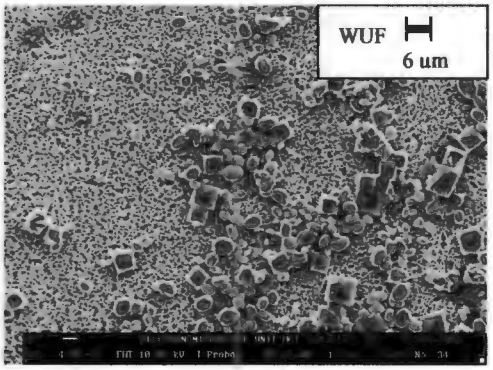


Figure 5.5: Scanning electron micrographs of crystals induced from the ultrafiltered urine of black subjects (BUF) before and after the addition of OPN at a final concentration of 0.5 mg/L at 4K magnification. Left panel shows BUF alone. Right panel shows BUF + OPN.



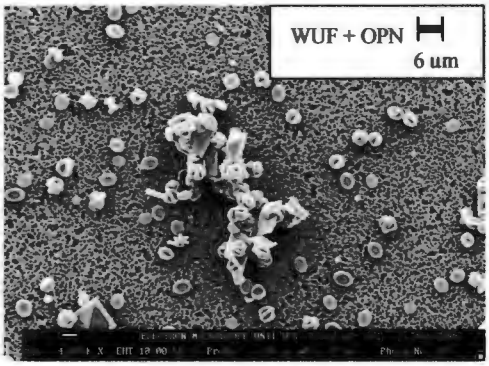


Figure 5.6: Scanning electron micrographs of crystals induced from the ultrafiltered urine of white subjects (WUF) before and after the addition of OPN at a final concentration of 0.5 mg/L at 4K magnification. Left panel shows WUF alone. Right panel shows WUF + OPN.

Comment

The observation of COM in BUF and a mixture of COM and COD in WUF is of great importance. As stated by Wesson et al. (1998), COM crystals have a lower epithelial cell binding affinity than COD crystals. The observations in BUF suggest that OPN acts as an inhibitor of nucleation (fewer particles) and as an inhibitor of either growth or aggregation (smaller sizes). On the other hand, the observation in WUF suggests that OPN promotes aggregation. Irrespective of the importance of these observations within the particular urine,

the results demonstrate that BUF and WUF elicit different responses in the role played by OPN, thereby highlighting a synergistic relationship between protein functionality and urine composition.

Effect of OPN on [^{14}C]-oxalate deposition

The mean effect of commercial OPN on ^{14}C-oxalate deposition in BUF (n = 5) and WUF (n = 5) is presented in Figure 5.7 (a) and (b), respectively. Addition of OPN to BUF resulted in a considerable decrease in the amount of precipitated ^{14}C-oxalate by 23 % relative to the control at 120 minutes. The protein also slightly reduced the amount of deposition in WUF. However, the decrease at 120 minutes was only 5 %. Individual plots are shown in Appendix 3, Figures 3.9 and 3.10.

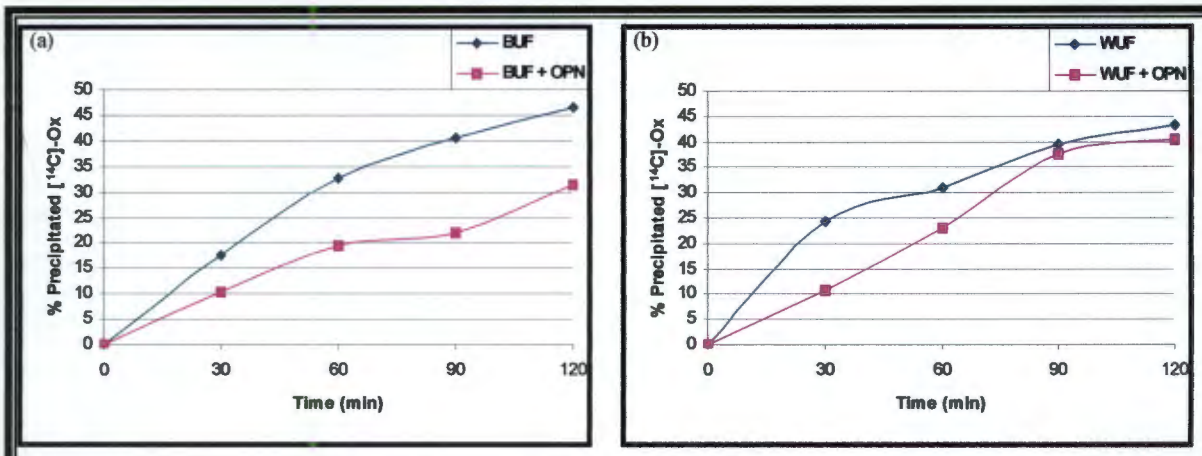


Figure 5.7: Mean percentage [^{14}C]-Oxalate deposited in (a) BUF and (b) WUF before and after the addition of OPN at a final concentration of 0.5 mg/l.

Comment

These results show that OPN is an inhibitor of CaOx crystallization nucleation. More importantly, however, the synergistic role of the two urines is again apparent, with BUF being more actively involved than WUF in supporting the inhibition process.

Effect of OPN on COM and COD crystal aggregation

The mean aggregation slopes and percentage inhibition of aggregation induced by each additive is shown in Table 5.4. Figure 5.8 shows the mean aggregation plots of a COM crystal slurry before and after the addition of BUF, WUF and OPN (n = 5 for all). Individual plots and aggregation slopes together with percentage inhibition of aggregation from which the means were calculated are given in Appendix 3, Figures 3.11 and 3.12 and Table 3.9. Addition of OPN to the COM slurry inhibited aggregation significantly (94 %, $p < 0.05$). When OPN was added in the presence of either BUF or WUF, inhibition dropped significantly. No difference was observed between BUF and WUF in this regard.

Table 5.4: Mean slopes of absorbance vs. time graphs, R^2 and percentage inhibition of aggregation (I_A %) of the COM crystal slurry after the addition of BUF, WUF and OPN (n= 5 for all).

Sample	Slope X -10^{-4} (OD/sec); R^2	I_A % (p-values , wrt COM)	p-value
COM	34; (0.992)		
COM + OPN	2.0; (0.974)	94 ($p < 0.05$)	
COM + BUF	25; (0.984)	26	
COM + BUF + OPN	21; (0.981)	38	
COM + WUF	17; (0.969)	50	
COM + WUF + OPN	20; (0.971)	41	
***BUF vs. WUF**			***0.051**
BUF vs. BUF + OPN			**0.147**
WUF vs. WUF + OPN			**0.730**
BUF + OPN vs. WUF + OPN			**0.882**

** indicates that the difference is statistically significant: $p \leq 0.05$*

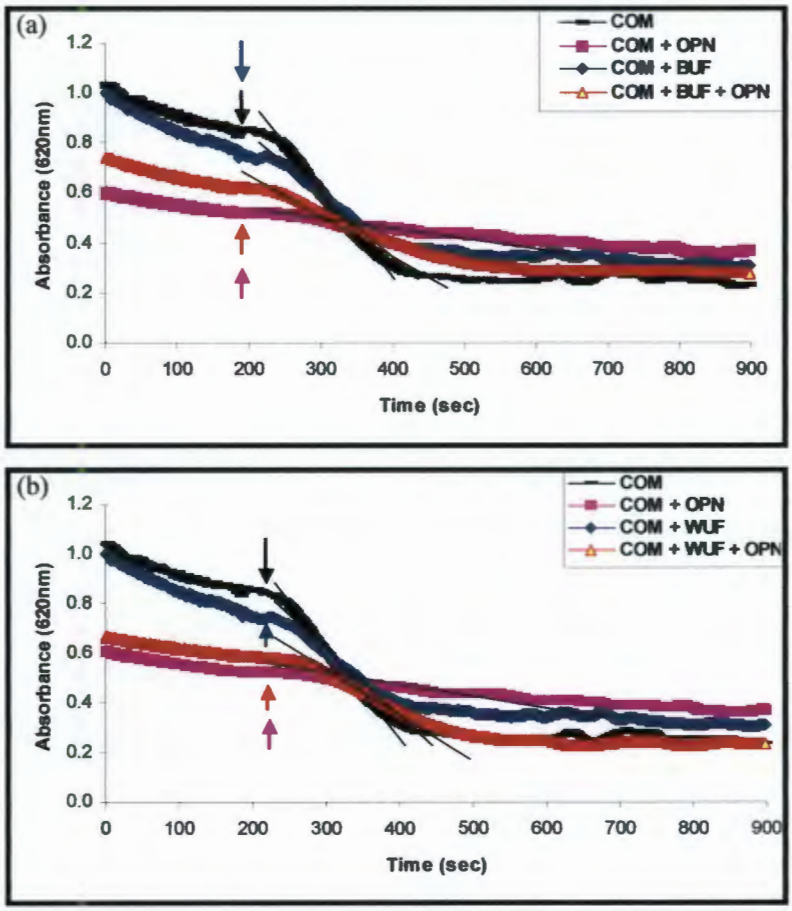


Figure 5.8: Mean COM crystal sedimentation plot of absorbance vs. time before and after the addition of (a) OPN, BUF and BUF + OPN and (b) WUF and WUF + OPN at a final concentration of 0.5 mg/L of OPN. An arrow indicates the time at which the stirrer was switched off and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Table 5.5 and Figure 5.9 show the absorbance slopes and percentage aggregation inhibition and the average plot for COD slurry before and after the addition of OPN (n = 5). Individual results are shown in Appendix 3, Figure 3.13 and Table 3.10. The addition of OPN to the COD slurry resulted in 42 % inhibition of aggregation. Attention is drawn to the fact that, this is 52 % lower than the 94 % inhibition induced by OPN in the COM slurry.

Table 5.5: Mean slopes of absorbance vs. time graphs, R^2 and percentage inhibition of aggregation (I_A %) of the COD crystal slurry after the addition of OPN (n = 5).

Sample	Slope X -10^{-4} (OD/sec); (R^2)	I_A % (p-value wrt COD)
COD	24; (0.993)	
COD + OPN	14; (0.931)	42 ($p < 0.05$)

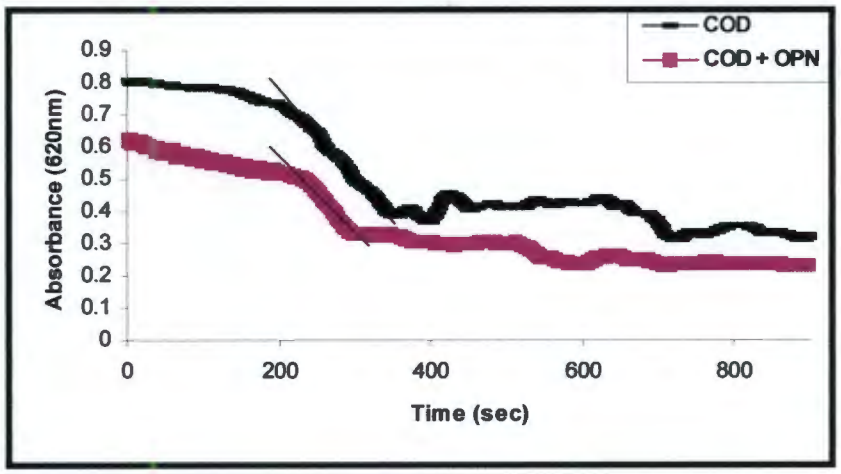


Figure 5.9: Mean COD crystal sedimentation plot of absorbance vs. time before and after the addition of OPN at a final concentration of 0.5 mg/L. An arrow indicates the time at which the stirrer was switched off was and the trendline indicates the absorbance slope used for the calculation of aggregation inhibition.

Comment

These experiments show that OPN is an inhibitor of both COM and COD crystal aggregation. Since inhibition of COD aggregation was not tested in the presence of BUF or WUF, it is not possible to deduce whether the extent of the inhibition is greater in the mono- or di-hydrate. However, the experiments involving the protein in the absence of urine suggest that OPN is a stronger inhibitor of COM crystal aggregation than it is of COD crystal aggregation. It is not possible to deduce from these data whether BUF and WUF play a synergistic role in this regard.

Effect of OPN on crystal nucleation

The average nucleation inhibition percentages (n = 5) are given in Table 5.6 and the corresponding mean plots of absorbance vs. time are shown in Figure 5.10. Individual plots and percentage inhibition of nucleation values are in Appendix 3, Figures 3.14 and 3.15 and Table 3.11. OPN significantly inhibited nucleation of CaOx crystals in an inorganic solution (95 %) and in urine from both black (95 %) and white (93 %) male subjects. The mixture of the protein and the urine from either race group inhibited nucleation to a similar extent as the protein (95 %) or urine on its own, BUF (95 %) and WUF (93 %).

Table 5.6: Mean slopes of absorbance vs. time graphs, R^2 values and percentage nucleation inhibition (I_N %) induced by OPN (n = 5).

Sample	Slope (X -10^{-4}; OD/sec); R^2	I_N % (p-values , wrt control)	p-values
Control (Ctrl)	43; (0.998)		
Ctrl + OPN	2.0; (0.982)	95 ($p < 0.05$)	
Ctrl + BUF	2.0; (0.993)	95 ($p < 0.05$)	
Ctrl + BUF + OPN	4.0; (0.996)	91 ($p < 0.05$)	
Ctrl + WUF	3.0; (0.995)	93 ($p < 0.05$)	
Ctrl + WUF + OPN	3.0; (0.994)	93 ($p < 0.05$)	
BUF vs. WUF			**0.188**
BUF vs. BUF + OPN			**0.172**
WUF vs. WUF + OPN			**0.518**
BUF + OPN vs. WUF + OPN			**0.477**

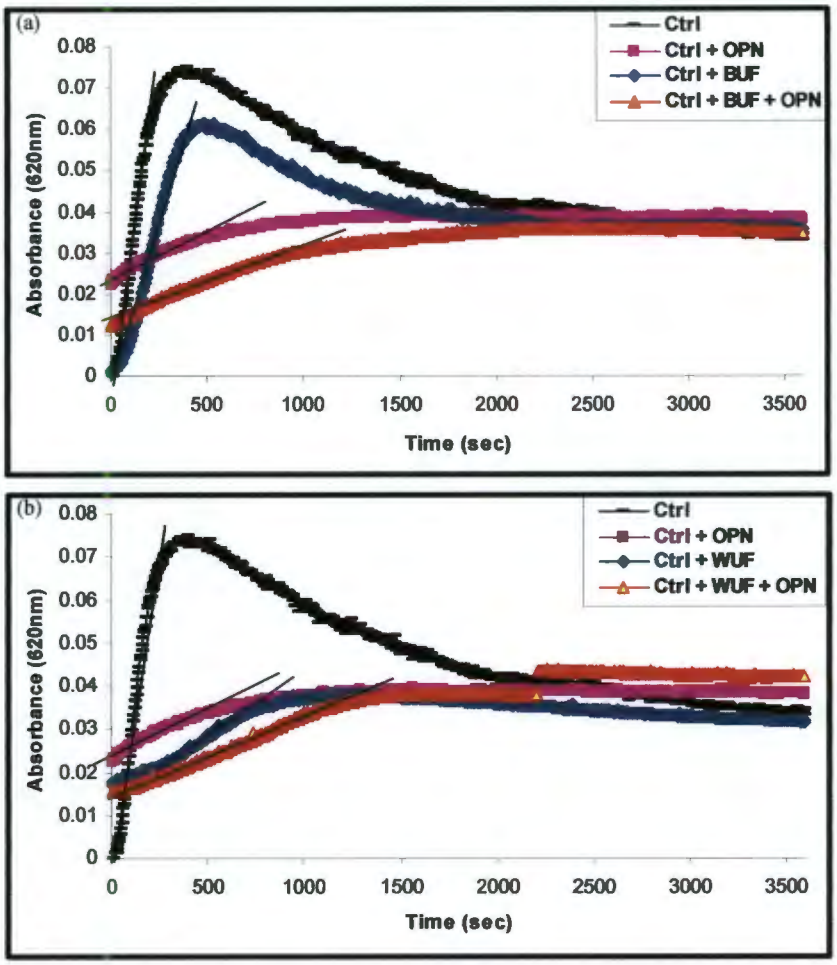


Figure 5.10: Mean CaOx crystal nucleation plots of absorbance vs. time before and after the addition of (a) BUF, OPN and BUF + OPN and (b) WUF, OPN and WUF + OPN at final concentrations of 0.5 mg/L of OPN. The trendline indicates the absorbance slope used for the calculation of nucleation inhibition.

Comment

These results provide strong evidence to support the hypothesis that OPN is an inhibitor of CaOx crystal nucleation. It is not possible to deduce the synergistic role (if any) of the respective urines.

Effect of OPN on COM crystal Zeta Potential

Table 5.7 shows the individual zeta potential values of the COM crystal slurry before and after the addition of OPN (n = 9). Figure 5.11 shows the histogram of the averaged values shown in Table 5.7. Addition of OPN to the COM slurry induced an increase in the magnitude of the negative zeta potential in six of the nine experiments. There was no significant difference between the mean values for zeta potential of the control and that of the protein-dosed slurry.

Table 5.7: Zeta potential of COM crystal before and after the addition of OPN (n = 9).

Number of runs	COM	COM + OPN	p- value
1	-10.3	-15.2	
2	-12.8	-11.1	
3	-15.3	-11.7	
4	-7.8	-5.97	
5	-10.8	-13.6	
6	-11.2	-13.6	
7	-12.5	-15.9	
8	-14.3	-16.6	
9	-14.8	-17.9	
Mean ± SE	**12.2 ± 1.02**	**13.6 ± 1.02**	**0.38**

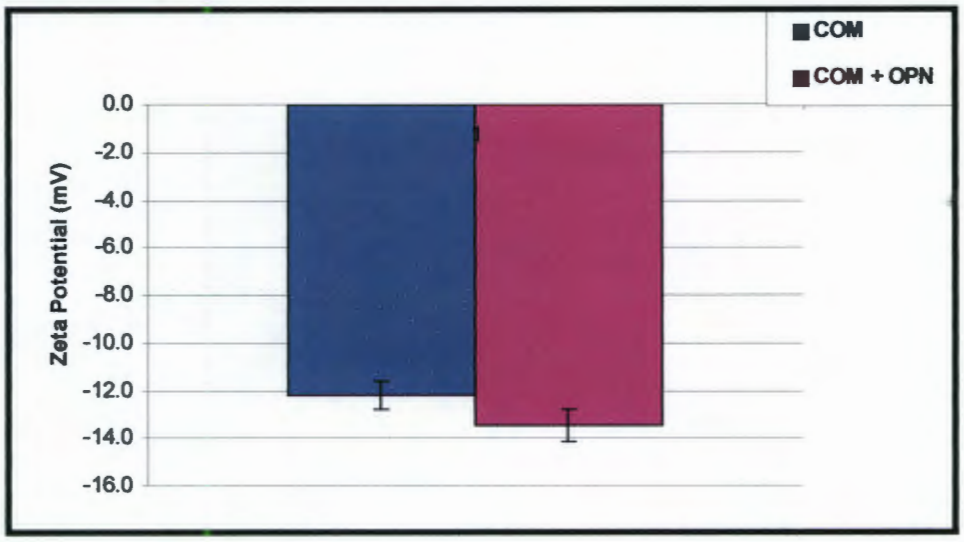


Figure 5.11: Mean zeta potential histogram before and after the addition of OPN to COM crystal slurry.

Effect of OPN on COD crystal Zeta Potential

Table 5.8 shows the individual zeta potential values of the COD crystal slurry before and after the addition of OPN (n = 9). Figure 5.12 shows the histogram of the averaged values shown in Table 5.8. Addition of OPN to the COM slurry induced a significant increase in the magnitude of the negative zeta potential as indicated by an increase in the negative charge.

Table 5.8: Zeta potential of COD crystal before and after the addition of OPN (n = 9).

Number of runs	COD	COD + OPN	p- value
1	-12.1	-12.4	
2	-14.9	-16.4	
3	-15.8	-19.2	
4	-10.5	-17.8	
5	-11.1	-14.8	
6	-9.27	-10.6	
7	-10.6	-10.5	
8	-12.1	-16.8	
9	-12.2	-17.3	
Mean ± SE	**-12.1 ± 0.90**	**-15.1 ± 0.90**	**0.03**

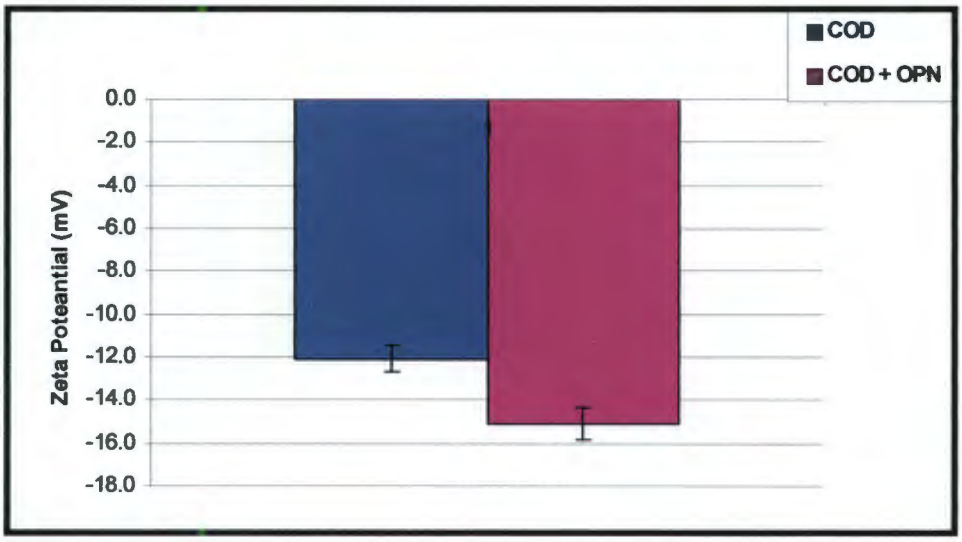


Figure 5.12: Mean zeta potential histogram before and after the addition of OPN to COD crystal slurry.

Comment

As explained earlier, an increase in the magnitude of the negative zeta potential signifies an increase in the electrostatic forces of repulsion and is therefore interpreted as an increase in the inhibition of aggregation. The addition of OPN to the COM slurry did not produce a significant change in the zeta potential indicating that the protein had no effect on aggregation inhibition. However, in the COD slurry, a significant change was observed, thereby suggesting an increase in aggregation inhibition. Thus, the zeta potential measurements have shown that OPN strongly inhibits this process in COD but not in COM. This is in marked contrast to the results of the aggregation experiment involving crystal sedimentation.

5.5 DISCUSSION

Composition data for the pooled urines demonstrated that phosphate, potassium and RS of brushite were significantly lower in urine samples from black subjects. The lower phosphate concentration in urine samples from black subjects is in agreement with the results published by Lewandowski et al. (2002). Contrary to the data obtained for individual urines in Chapter 3, RS of CaOx was significantly higher in samples from black subjects. Considering the low incidence of kidney stone formation in the black population, the high RS of CaOx in samples from black subjects is puzzling. However, comparison of compositional data from pooled urines with individual urines is likely to yield discrepancies because components in any particular aliquot may dominate. These discrepancies might be also due to considerable variations in individual samples.

As explained in Chapters 3 and 4, the Coulter Counter has limitations with regard to its ability to distinguish between a single particle and an aggregate. Aggregates which contain empty spaces between them are recorded by the instrument as if they are large particles, giving erroneously high crystal volume deposition (Ryall et al. 1995). Thus the Coulter Counter data proved difficult to interpret. Despite these limitations, OPN appeared to reduce the number, volume and rate of particle formation in both BUF and WUF. It is important to note that the addition of OPN in BUF and WUF induced a favourable reduction in particle size by 19 % and 10 %, respectively. Since particle size is a determinant of the likelihood of crystal retention in the renal tubular cells (Kok and Khan 1994), the aforementioned reduction in size is a significant observation.

The precipitation of COD in WUF as shown by scanning electron microscopy is not surprising considering the high (but not significantly different) urinary calcium concentration in the white group reported in this study and others (Modlin 1967; Whalley et al. 1998; Rodgers and Lewandowski 2002) and the fact that COD precipitates at very high calcium concentrations (Webber et al. 2003, Ryall et al. 2005). An aggregate of COM and COD crystals resulting after the addition of OPN to WUF is an indication of promotion of aggregation in the urine of the white race group. Nonetheless, precipitation of COD crystals in WUF before and after the addition of OPN could be of considerable inhibitory benefit since COD crystals have been reported to be less adherent to renal epithelial cells (Wesson et

al, 1998). ^{14}C-oxalate deposition data were also supportive of the above results that OPN inhibits CaOx crystal nucleation and that BUF is superior in this regard compared to WUF.

The role of OPN in crystal nucleation and aggregation was also investigated to supplement the ^{14}C-oxalate deposition data. OPN significantly inhibited both CaOx crystal nucleation and aggregation. A comparative study of COM and COD crystal aggregation by OPN demonstrated a high inhibition of COM crystal aggregation (94 %) compared to COD crystal aggregation (42 %). However it was not possible to deduce the synergism between OPN and the ultrafiltrates from both groups.

The aggregation inhibition data were supported by the measurement of the magnitude of the zeta potential (more negative charge). Addition of OPN to both COM and COD crystal slurries induced an increase in the negative charge. However, the significantly higher negative charge on COD crystals (-15.1 mV) compared to that on COM crystals (-13.6 mV) is noteworthy. This is an indication of high crystal disaggregation by COD crystals. As reported by several authors (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005) and as shown in Chapter 4, OPN is the main intracrystalline protein in COD crystals and thus the high disaggregation by COD compared to COM could be attributed to the preferentially higher inclusion of this protein in the former. COD crystals have a higher positive charge which is due to the high concentration of calcium ions which results in high repulsion between crystals and thus favours disaggregation (Cerini et al. 1999). OPN has also been reported to have a high calcium binding affinity due to the presence of the RGD sequence (Kohri et al. 1990). This high calcium affinity together with the high positive charge of COD could be the main contributing factor to the significant repulsion observed from the zeta potential.

The crystallization data reported in this study demonstrated that OPN is an inhibitor of CaOx crystal nucleation, growth and aggregation to a greater extent in BUF than WUF. OPN may therefore be a superior provider of protection against CaOx crystallization in the urine of black subjects compared to that of whites, thereby contributing to the low stone incidence in the black group.

CHAPTER SIX

THE EFFECT OF SODIUM CHLORIDE, SODIUM BICARBONATE AND SODIUM CITRATE ON THE URINARY RISK FACTORS FOR CALCIUM OXALATE KIDNEY STONE FORMATION AND ON BONE TURNOVER MARKERS

6.1. INTRODUCTION

Excessive dietary sodium intake is associated with elevated blood pressure. Good blood pressure control has been linked with improvements in cardiac, cerebral and kidney function and in reduction in morbidity and mortality from cardiovascular and renal disease (Peterson et al. 1995). Dietary sodium is a well-known determinant of urinary calcium excretion (Burtis et al. 1994). Reducing dietary salt (sodium) helps to reduce the amount of calcium in the urine, which in turn reduces the tendency for calcium stone formation (King et al. 1964; Muldowney et al. 1982; Shortt and Flynn 1990; Shortt et al. 1998). Dietary salt reduction is best accomplished by not adding salt to food and by avoiding high sodium foods such as processed meats, salty convenience foods (such as canned soups, noodle or rice mixes) and salty snacks. Even a moderate reduction of dietary sodium can reduce not only hypertension but also hypercalciuria, thereby preventing kidney stones and osteoporosis (Nordin et al. 1992; Goulding 1990).

Calcium has a high influence on skeletal development during growth and therefore is an important determinant of peak bone mass (Matkovic et al. 1995). Maximal positive balance between calcium intake and obligatory losses of calcium is required for the attainment of peak bone mass (Matkovic et al. 1995). High losses of calcium in urine reduce calcium accumulation into bone, which may have a negative impact on skeletal development and ultimately on bone loss. As mentioned earlier in the main introduction chapter, it has been estimated that a 100 mmol increment in daily sodium intake is associated with an average loss of urinary calcium of approximately 1 mmol/24 hours (Nordin et al. 1993). Calcium/sodium ratios (1 mmol Ca/100 mmol Na) of more than 1 in normal living populations (no diet restrictions) of postmenoupausal women have been reported by

numerous researchers (Goulding et al. 1986; Nordin et al. 1993; Evans et al. 1997; Ginty et al. 1998; Massey 2005).

These ratios can have values of 1.2 (Nordin et al. 1993; Ginty et al. 1998), 2.2 (Goulding et al. 1986), 3.0 (Massey 2005) and 3.7 (Evans et al. 1997). The dependence of urinary calcium excretion on sodium intake is attributable to the existence of linked common reabsorption pathways of both calcium and sodium ions in the renal tubular system (Matkovic et al. 1995; Ginty et al. 1998).

Of great interest is a study by Morris and Sebastian (2002) on alkali therapy in renal tubular acidosis. Acidosis is a renal tubular disorder caused by a restriction in reduction of urinary pH and titration of urinary buffers thus leading to the urinary excretion of acid. Metabolic acidosis is known to induce hypercalciuria and consequent negative calcium balance. It may also give rise to osteoporosis and other disorders of bone mineralization (Harrington et al. 1983). It does so by enhancing osteoclastic activity and inhibiting osteoblastic functioning and hence bone formation (Lemann et al. 1996). Conversely, metabolic alkalosis decreases bone calcium efflux by suppressing osteoclasts and stimulating osteoblasts (Bushinsky 1996). Osteoblasts are cells that mediate the formation of new bone and osteoclasts cause bone resorption. Osteoporosis occurs when bone resorption exceeds bone formation.

Several authors have investigated bicarbonate and citrate as therapeutic treatments of osteoporosis. Sodium bicarbonate ($NaHCO_3$) as a supplement to alkali therapy of renal tubular acidosis was used by several researchers and was proven to increase plasma bicarbonate and reversely induce net renal acid excretion (Sakhaee et al. 1983; Lutz 1984; Lemann et al. 1989). This resulted in an improved external balance of calcium as demonstrated by reductions in the urinary excretion rate, followed by an increase in serum concentrations of osteocalcin and a decrease in urine hydroxyproline. This investigation was followed by that of Sebastian et al. (1994) who also demonstrated an increase in serum osteocalcin and a decrease in urinary hydroxyproline.

As explained in Chapter 1, citrate has been reported to be a powerful therapeutic and prophylaxis alkali therapy in preventing the formation of stones which it accomplishes by forming soluble complexes with calcium resulting in low urinary excretion of calcium, and

inhibition of nucleation and agglomeration of CaOx crystals (Kok et al. 1986; Pak 1994; Allie-Hamdulay and Rodgers 2005). Preminger et al. (1985 (a); 1987) showed that citrate alkali therapy increased plasma bicarbonate which led to an increase in bone mineral density. Prior to this investigation, there was a report by Osther and co-workers (1993) that serum osteocalcin and urinary hydroxyproline were significantly decreased and increased, respectively, in kidney stone formers with renal tubular acidosis. Weger et al. (1999) in their study reported that patients who suffered from renal tubular acidosis, demonstrated symptoms of osteroporosis and some had either kidney stones or nephrocalcinosis (high concentration of calcium in the nephron). This justifies profound relationships between kidney stone disease and osteoporosis.

Despite the reported high dietary sodium intake by the black South African population, it is surprising that they do not have elevated urinary calcium levels (Modlin 1967, Whalley et al. 1998). As mentioned earlier in Chapter 1 (Introduction), other puzzling anomalies occur in the renal handling of various dietary and supplemental challenges in black subjects, prompting speculation that these mechanisms are different in black and white South African subjects (Lewandowski et al. 2001; Rodgers and Lewandowski 2002). These factors, together with the observations reported earlier in this chapter that salts such as sodium citrate (Sebastian et al. 1994; Bushinsky 1996; Ettinger et al. 1997; Laube et al. 2002 (a); Sellmeyer et al. 2002) and sodium bicarbonate (Lutz 1984; Lemann et al. 1989; Sebastian et al. 1994, 2005; Frassetto et al. 2005; Sakhaee et al. 2005) have inhibitory effects on CaOx stone formation and osteoporosis provided motivation for the study described in this chapter.

6.2 AIMS AND OBJECTIVES

- To determine the effect of dietary salt (NaCl) ingestion on the urinary physicochemical risk factors for calcium oxalate stone formation in black and white healthy male South African subjects with a view to establishing whether the renal handling of this agent is different in the two groups.

- To investigate the effect of the independent ingestion of sodium bicarbonate and a sodium citrate preparation on the urinary risk factors for CaOx stone formation in healthy black and white South African male subjects and to compare these parameters with those elicited by sodium chloride (NaCl) ingestion.

- To determine the effect of the ingestion of these salts on the CaOx crystallization properties of urine in healthy black and white South African male subjects.

- To determine the effect of the ingestion of the aforementioned salts on serum levels of osteocalcin (bone formation marker) and on urinary levels of deoxypyridinoline (bone resorption marker) in healthy black and white South African male subjects.

6.3 MATERIALS AND METHODS

6.3.*1* Study Population and Sample Size

20 black and 19 white, age matched male volunteers were recruited for the study from the University of Cape Town student community via advertisement. The sample size was based on the degrees of freedom for error for each race group as calculated statistically.

Inclusion Criteria

Healthy male subjects in the age range 18-30 years without any family history of renal or kidney stone disease were recruited for the study.

Exclusion Criteria

Subjects were excluded if they had:

- Family history of renal or kidney stone disease.
- Urinary tract infections during the course of the study or in the previous year from the start of the study.
- Hypertension, diabetes, liver disease, water retention and heart failure.

Subjects who were on medication such as antibiotics, supplements and vitamins were also excluded. Participants were asked not to take alcohol while participating in the study.

6.3.*2* Study Design

The study was divided into four experimental periods/protocols for each race group, each consisting of seven consecutive days. A Latin Square Design was followed for the random assignment of participants to sequences of protocols as shown in Table 6.1. This design was implemented for 20 black and 19 white subjects.

The four protocols followed were as follows: **Protocol L**; Low NaCl salt, 3 g/ day (Cerebos Ltd. SA;), **Protocol H**; High NaCl, 9 g/day (Cerebos Ltd.), **Protocol C**; Sodium bicarbonate, 6 g/ day (Allied Drug Co. SA) and **Protocol D**; Sodium citrate, 16 g/day (Citro-Soda*, Adcock Ingram SA).

***: Composition of Citro-soda (4 g sachet):**

1716 mg	sodium bicarbonate
858 mg	tartaric acid
702 mg	citric acid
613 mg	sodium citrate
111 mg	sugar

Table 6.1: Sequences followed by 20 subjects for four different protocols: **L**; low NaCl, **H**; high NaCl, **C**; sodium bicarbonate and **D**; sodium citrate.

	SUBJECT/SEQUENCE											
PERIOD/PROTOCOL	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	L	H	C	D	L	H	C	D	L	H	C	D
2	H	L	D	C	C	D	L	H	D	C	H	L
3	C	D	L	H	D	C	H	L	H	L	D	C
4	D	C	H	L	H	L	D	C	C	D	L	H

	SUBJECT/SEQUENCE							
PERIOD/PROTOCOL	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	L	H	C	D	L	H	C	D
2	H	L	D	C	C	D	L	H
3	C	D	L	H	D	C	H	L
4	D	C	H	L	H	L	D	C

This study was approved by the Clinical Research Ethics Committee of the University of Cape Town. Before participation, all subjects underwent a medical examination to assess if they were medically fit and healthy enough to ingest the salts. They all signed an informed consent form.

6.3.3 Protocol

(a) Salt administration and diet control

A summary of the protocol that was followed by each subject while participating in the study is shown in Figure 6.1.

Day 0: Collect baseline 24 hr urine sample.

Day 1: Deliver **Day 0** urine sample, blood sample taken.

Day 2:

Day 3: Take salt **(L/H/C/D)** with self – selected standardized diet.

Day 4:

Day 5: Collect a 24 hr urine sample.

Day 6: Deliver **Day 5** urine sample.
Take salt **(L/H/C/D)** with controlled diet.

Day 7: Collect a 24 hr urine sample.

Day 8: Deliver **Day 7** sample, blood sample taken before breakfast.

Day 8 – Day 14: Washout period.

Day 15: Start on the next salt regimen **(L/H/C/D)** and follow the same schedule.

Figure 6.1: A summary of the protocol followed by each subject while participating in the study.

L: Low NaCl, H: High NaCl; C: Sodium bicarbonate; D: Sodium citrate

Prior to any restriction in diet and salt content, a 24-hour baseline/control urine sample was collected **(Day 0)**. On **Day 1** a blood sample was taken and collected into EDTA-free tubes (to avoid complexation with calcium) by a professional nurse on **Day 1** before the ingestion of the salt. All the results obtained after the ingestion of the salts were compared to the control sample. Each participant/subject followed each protocol **(L/H/C/D)** and controlled/standardized diet for seven days. This consisted of the first five days **(Day 1-Day 5)** on a self-selected standardized diet (where they had to eat a limited number of portions for each food type a day, Table 6.2) while taking the assigned salt **(L/H/C/D)** and the last two days **(Day 6 - Day 7)** on a strictly controlled diet shown in Tables 6.3 and 6.4 with strictly controlled nutrients, with all foods and water provided. Subjects were requested to collect urine samples on **Day 5** and **Day 7**, and when delivering the samples on **Day 8**, a professional nurse took a second blood sample. There was a washout period of seven days **(Day 8 – Day 14)** after each protocol. The blood samples were centrifuged at 1500 X g for 15 min, and a supernatant/serum was frozen at -80 ^{o}C within two hours of collection. Aliquots (5 mL) of **Day 0** and **Day 7** urine samples were also kept at -80 ^{o}C. The blood samples were used to analyze for osteocalcin (bone formation marker) and the frozen urine samples were used for deoxypyridinoline (DPD) analysis using an Enzyme Linked Immunosorbent Assay (ELISA).

Table 6.2 shows the self-selected standardized diet that the subjects followed for the first five days **(Days 1-5)** of each protocol. The daily allowance indicates the number of portions permitted from the listed food groups per day. Each item in the 'portion size' column is an example of 1 portion. Subjects were also instructed to drink 2.2 L of water each day.

Table 6.2: Self-Selected Standardized Diet Sheet for 50 mmol/day salt (low salt). To achieve high salt levels, subjects were given 9 g of table salt a day.

Daily Allowance	Portion Size (1 Portion examples)
2 milk portion	1 cup milk 1 cup yoghurt etc
Choose any 5 fat portions	1 tsp butter 1 tsp margarine 1 tsp sunflower, canola or olive oil 1 tsp mayonnaise ¼ of a avocado pear 2 tsp peanut butter etc
Choose any 2 vegetable portions	½ cup cooked vegetables 1 cup green fresh salad * see critical food list etc
Choose any 2 fruit portions	any medium-sized fruit ½ cup fruit juice * see critical food list etc
Choose any 12 starch portions	1 slice of bread ½ cup cooked porridge ½ cup cereal 1 weetbix 3 provitas 1 medium / 2 small potatoes ½ cup cooked rice, samp, pasta ½ cup legumes (eg lentils, beans, chickpeas, soya) etc
Choose any 5 protein portions	30g meat/chicken/fish = 1matchbox 45g cottage cheese = 1 heaped Tbsp 30g hard cheese = 1 matchbox 2 cheese wedges/4tsp cheese spread 1 egg etc
Choose any 3 sugar portions	1 tsp sugar 200ml pudding 50ml cool drink 2 blocks chocolate 2 Jelly Beans etc

Table 6.3 shows the controlled standardized diet that the subjects had to follow for the last two days **(Days 6 and 7)** of each protocol. All the meals were provided by the investigator. Subjects were instructed to eat and drink only what was provided, nothing else.

Table 6.3: Composition of the 2-day **(Day 6 and 7)** controlled standardized diet for all the salts. To achieve high salt levels, subjects were given 9 g of table salt a day.

Meal	Day 6	Day 7
Breakfast	2 bread rolls (100 g) 8 g unsalted butter 1 X 15 g apricot jam 140 g mushroom sauce† 200 ml coffee + 40 ml milk (2 %)	2 bread rolls (100 g) 8 g unsalted butter 1 X 15 g apricot jam 140 g mushroom sauce† 200 ml coffee + 40 ml milk (2 %)
Snack 1	1 muesli bar (Jungle energy bar – Berries, 40 g) ½ cup fruit salad	1 muesli bar (Jungle energy bar – Berries, 40 g) ½ cup fruit salad
Lunch	145 ml cheese sauce* 120 g mixed vegetables + 10 g unsalted butter 120 g pasta	145 g cheese sauce* 120 g mixed vegetables + 10 g unsalted butter 120 g pasta
Snack 2	1 medium apple (100 g) 175 g yoghurt with fruit (low fat)	1 medium apple (100 g) 175 g yoghurt with fruit (low fat)
Supper	2 boiled potatoes (190 g) 1 slice of whole-wheat bread 8 g unsalted butter 2 X 16 g cream cheese (Simonsburg) 1 tomato (50 g) + 20 g cucumber 1 apple (100 g)	2 boiled potatoes (190 g) 1 slice of whole-wheat bread 8 g unsalted butter 2 X 16 g cream cheese (Simonsburg) 1 tomato (50 g) + 20 g cucumber 1 apple (100 g)
Late meal	1 slice of whole-wheat bread 15 g peanut butter	1 slice of whole-wheat bread 15 g peanut butter

Beverages (2.2 l/day):

200 ml coffee (2 g decaffeinated coffee powder sachet in 200 ml hot water)
700 ml low mineral content water until 13h00
600 ml low mineral content water until 17h00
700 ml low mineral content water until 21h00
(Composition of low mineral water: Ca: 6.1 mg/L; Mg: 1.0 mg/L; Na: 17 mg/L).

† mushroom sauce per person: 18 g unsalted butter, 30 g onion, 180 g button mushrooms, 50 g crème, 5 g parsley
* cheese sauce per person: 13 g unsalted butter, 13 g flour, 16 g cheddar cheese (2 slices), 125 ml milk (2 %)

Table 6.4 shows the total amount of the controlled food constituents in **Day 6** and **Day 7** standardized diet.

Table 6.4: Controlled constituents in the standardized diet.

Description	Total Amount
Energy (kJ)	10139
Total protein (g)	57.3
Total fat (g)	97.9
Carbohydrate (g)	298
Ca (mg)	800
Na (mg)	1290
Cl (mg)	1898
Citric acid (mg)	2417
Oxalic acid (mg)	48

(b) Urine Analysis

All the urinary parameters listed in Chapter 2 were analyzed in all the samples. In addition, bicarbonate, ammonia and free calcium were also analyzed. Bicarbonate was determined using standard titrimetic methods (de Andrade et al. 2005) while an ammonia electrode (IS 570-NH_3, Metrohm, Switzerland) was used for determination of ammonia. A calcium ion specific electrode (Metrohm, Herisau Switzerland) was used for the measurement of free calcium.

(c) Urine physicochemical risk factors

The computer program, EQUIL2 was used to determine the relative supersaturation of brushite, CaOx and uric acid (Werness et al. 1985). The Tiselius Risk Index (TRI) was also calculated (Tiselius 1982). In addition, the BONN-Risk-Index (BRI), (a standardized in-vitro test used to assess the crystallization risk of calcium oxalate in urine) was also measured (682 Photometer, Metrohm, Herisau Switzerland) (Laube et al. 2002 (b)).

6.3.4 Statistical Analysis

Statistical analysis was performed by an Analysis of Variance (ANOVA) using a GenStat computer programme. In considering comparisons between diets, the Bonferroni technique was used at the 5 % level of significance.

6.3.5 Crystallization experiments

Urine collection, treatment and determination of the CaOx MSL

Twenty-four hour urine samples were collected in plastic bottles without preservatives and filtered before use (0.75 μm pre-filter and 0.45 μm nitrocellulose) as described previously in Chapter 2. The CaOx metastable limit was determined using the Coulter Counter as previously described in Chapter 2.

Particle formation kinetics

The particle number, volume and size were determined as a function of time using the Coulter Counter as previously described in Chapter 2.

Scanning Electron Microscopy (SEM)

Crystals which formed after the kinetics experiment were examined by scanning electron microscopy as described in Chapter 2.

^{14}C-oxalate crystal deposition kinetics

This experiment was performed as previously described in Chapter 2.

CaOx crystal aggregation

Before this experiment was conducted, COM crystals were prepared as described in Chapter 2 using the method of Pak et al. (1975). The percentage inhibition of aggregation was also determined as described in Chapter 2.

CaOx crystal nucleation assay

This experiment was conducted following the method developed by Hess et al. (1995). The absorbance was measured at 620 nm and the percentage inhibition of nucleation was determined.

6.3.6 Analysis of Deoxypyridinoline (DPD) in urine using Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of DPD in urine was determined using a competitive ELISA kit (Metra Biosystems, USA) with 96 well plates already coated with DPD. All the reagents, solutions and buffers were provided by the manufacturers. Firstly, a calibration curve of absorbance vs. DPD concentration was constructed using the range of standard solutions in the kit (0, 3, 10, 30, 100, 300 nmol/L). In each case, 25 µL of each standard concentration (in duplicate) was diluted 10 times with the assay buffer (noionic detergent in a buffered solution). 50 µL of each dilute standard was added to each well, followed by the addition of 100 µL of the enzyme conjugate (alkaline phosphatase). The wells were covered with the provided cover tape and incubated for 2 hours at 4 °C. The wells were then washed 3 times with 250 µL of ice-cold wash buffer followed by incubation with 150 µL of working substrate solution (*p*-nitrophenyl phosphate) for 60 minutes at room temperature. 100 µL of 0.5 N NaOH was added to stop the reaction and the absorbance was read at 405 nm using an ELISA plate reader (Anthos LabTech Instrumnents, Australia). The resulting calibration curve is shown in Figure 6.2. Urine samples were then treated in the same way for the determination of their DPD concentrations.

6.3.7 Analysis of osteocalcin (OC) in serum using Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of osteocalcin in serum was determined using a competitive ELISA kit (Metra Biosystems, USA), with 96 well plates already coated with osteocalcin. All the reagents, solutions and buffers were provided. Similarly to DPD determination, a calibration curve was first plotted. 25 µL of increasing concentrations of the standards (0, 2, 4, 8, 16 and 32 ng/mL) were added to each of the coated wells. This was done in duplicate for each of the standards. All the incubations were carried out at room temperature. An anti-osteocalcin antibody (125 µL) was added to each well (that already contained the standard) and incubated for 2 hours at 25 °C. This was followed by 3 washes of each well with 300 µL of 1X concentrated wash buffer. Each well was incubated in 150 µL of the enzyme conjugate (IgG alkaline phosphatase) for 1 hour. The wells were then washed 3 times with 300 µL of the wash buffer. After the third wash, the wells were vigorously blotted dry on paper towels. The dried wells were incubated in a working substrate solution for 40 minutes. The reaction was stopped by the addition of 5 M NaOH and the absorbance was read at 405 nm using an ELISA plate reader (Anthos LabTech Instrumnents, Australia). A calibration curve was plotted and is shown in Figure 6.3.

For the determination of the concentration of osteocalcin in serum, the same procedure as above was followed and the same quantities of reagents and buffers were used.

6.3.8 Deoxypyridinoline (DPD) and Osteocalcin (OC) Calibration Curves

Figure 6.2 and 6.3 below represent the standard calibration curves for deoxypyridinoline (DPD) and osteocalcin (OC), respectively from which the concentrations of urinary DPD and serum OC were determined.

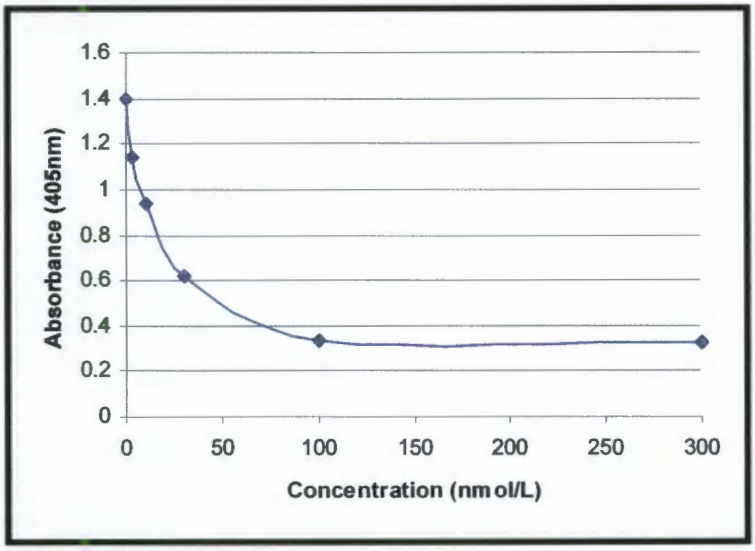


Figure 6.2: Calibration curve for deoxypyridinoline (DPD).

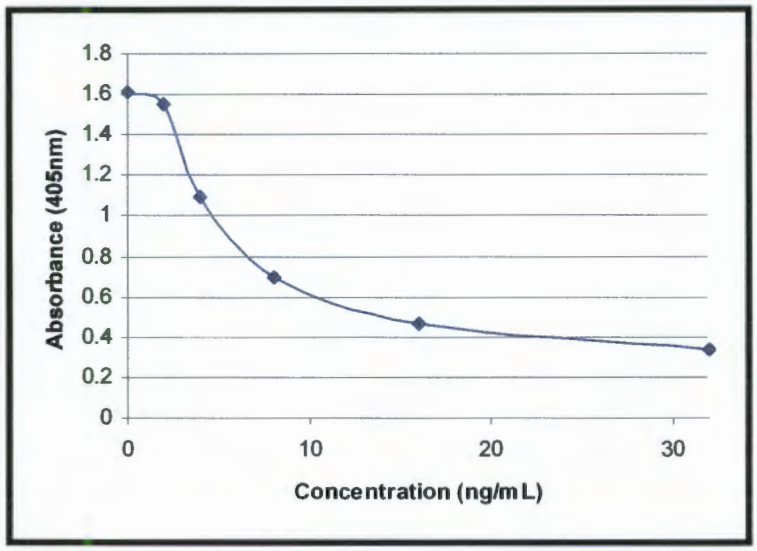


Figure 6.3: Calibration curve for osteocalcin (OC).

6.4 RESULTS

6.4.*1* Urine composition and physicochemical properties of urine

The mean composition and physicochemical parameters of urine samples from black (n = 20) and white subjects (n = 19) at baseline and at day 5 and 7 of each protocol are presented in Tables 6.5 to 6.13. Individual parameters are presented in Appendix 4, Tables 4.1 and 4.2. A p-value of ≤ 0.05 with a (*) indicates a statistically significant difference.

Table 6.14 shows a summary of common and different physicochemical changes in samples from both groups after each protocol.

At baseline, the BRI, RS of CaOx and RS of brushite were significantly lower in blacks than whites (Table 6.5). For all the protocols, significant changes were observed on day 7 samples than on day 5. There was also a significant increase in volume of urines from both groups at day 7 of each protocol.

The low NaCl protocol did not result in any changes in urine parameters on day 5 but following the strictly controlled diet on day 7, a significant beneficial decrease in uric acid and free calcium was observed in blacks. For whites, there was a significant decrease in RS of brushite, CaOx and uric acid on day 7 of this protocol.

As expected there was a significant increase in sodium and chloride after administration of high NaCl protocol. A significant and unexpected decrease in free calcium and RS of uric acid was also observed in samples from black subjects after this protocol whereas a significant decrease in RS of brushite and CaOx was depicted in samples from whites.

A significant beneficial increase in pH, calcium, citrate, free calcium, BRI and RS of uric acid was revealed in both groups after administration of sodium bicarbonate and sodium citrate. A favourable decrease in RS of CaOx was also observed in samples from white subjects following the ingestion of these two protocols.

Table 6.5: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at baseline. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Parameter	B	W	p-values
pH	6.13 ± 0.107	6.12 ± 0.107	
Volume (mL/24hr)	1328 ± 143	1322 ± 143	
Ammonia (mg/L)	20.7 ± 4.16	20.9 ± 4.16	
Bicarbonate (mmol/L)	1.45 ± 0.314	1.032 ± 0.314	
*Calcium (mmol/24hr)	2.28 ± 0.339	3.75 ± 0.339	**< 0.05***
Citrate (mmol/24hr)	2.60 ± 0.282	2.29 ± 0.282	
Chloride (mmol/24hr)	139 ± 8.72	135 ± 8.72	
Creatinine (mmol/24hr)	13.8 ± 0.959	15.6 ± 0.959	
Magnesium (mmol/24hr)	2.29 ± 0.375	3.13 ± 0.375	
Oxalate (mmol/24hr)	0.329 ± 0.0257	0.343 ± 0.0257	
Phosphate (mmol/24hr)	23.4 ± 2.68	27.5 ± 2.68	
Potassium (mmol/24hr)	44.1 ± 6.72	60.3 ± 6.72	
Sodium (mmol/24hr)	117 ± 15.1	109 ± 15.1	
Sulphate (mmol/24hr)	18.8 ± 2.09	18.1 ± 2.096	
Uric acid (mmol/24hr)	3.06 ± 0.249	3.31 ± 0.24	
Free Calcium (Ca^{2+}, mmol)	0.402 ± 0.0467	0.494 ± 0.0467	
BONN-Risk-Index (BRI)	1.02 ± 0.161	1.74 ± 0.161	**< 0.05***
Tiselius Risk Index (TRI)	285 ± 35.6	358 ± 35.6	
*RS Brushite	0.547 ± 0.343	1.51 ± 0.343	**< 0.05***
*RS CaOx	2.34 ± 0.568	5.87 ± 0.568	**< 0.05***
RS Uric acid	1.87 ± 0.436	2.18 ± 0.568	
CaOx MSL (mmol/L)	90 ± 10	75 ±10	

**Difference is statistically significant: p ≤ 0.05, B (Urine samples from black subjects); W (Urine samples from white subjects). RS: Relative Supersaturation.*

Table 6.6: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 5 of protocol L (low NaCl). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	Low NaCl	p > 0.05	Baseline	Low NaCl	P> 0.05	Low NaCl p
pH	6.13± 0.084	6.13±0.084		6.12±0.112	6.33±0.112		
Volume (mL/24hr)	1328±167	1645±167		1322±124	1560±124		
Ammonia (mg/L)	20.7±1.58	24.2±1.58		20.9±5.87	33.6±5.87		
Bicarbonate (mmol/L)	1.45±0.414	1.52±0.414		1.03±0.290	0.825±0.290		
*Calcium (mmol/24hr)	2.28±0.327	1.97±0.327		3.75±0.523	3.39±0.523		**<0.05***
Citrate (mmol/24hr)	2.60± 0.237	2.78± 0.237		2.29±0.415	2.63±0.415		
Chloride (mmol/24hr)	139±11.5	148±11.5		135±15.3	133±15.3		
Creatinine (mmol/24hr)	13.8±0.960	13.8±0.960		15.6±1.48	14.3±1.48		
Magnesium (mmol/24hr)	2.29±0.327	2.37±0.327		3.13±0.398	2.67±0.398		
Oxalate (mmol/24hr)	0.329±0.0296	0.316±0.0296		0.343±0.0269	0.335±0.0269		
Phosphate (mmol/24hr)	23.4±2.55	21.0±2.55		27.5±2.88	28.1±2.88		
Potassium (mmol/24hr)	44.1±0.698	42.3±0.698		60.3±7.89	54.7±7.89		
Sodium (mmol/24hr)	117±21.3	132±21.3		109±19.9	111±19.9		
Sulphate (mmol/24hr)	18.8±2.22	18.6±2.22		18.1±1.99	20.8±1.99		
Uric acid (mmol/24hr)	3.06±0.222	3.11±0.222		3.31±0.296	3.43±0.296		
Free Calcium (Ca^{2+}, mmol)	0.402±0.099	0.289±0.099		0.481±0.0748	0.419±0.0748		
*BONN-Risk-Index (BRI)	1.02±0.176	0.768±0.176		1.69±0.308	1.50±0.308		**<0.05***
Tiselius Risk Index (TRI)	285±39.8	259±39.8		350±36.5	340±36.5		
*RS Brushite	0.547±0.123	0.412±0.123		1.51±0.362	0.779±0.362		**<0.05***
*RS CaOx	2.34±0.363	2.53±0.636		5.87±0.609	3.94±0.609		**<0.05***
RS Uric acid	1.87±0.386	1.68±0.386		2.18±0.417	1.41±0.417		
CaOx MSL (mmol/L)	90±8.39	60±8.39		75±12.4	75±12.4		

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.7: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 7 of protocol L (low NaCl). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	Low NaCl	P	Baseline	Low NaCl	p	Low NaCl p
pH	6.13±0.116	6.15±0.116		6.12±0.104	6.35±0.104		
*Volume (mL/24hr)	1328±161	2055±161	**< 0.05***	1322±105	1787±105	**< 0.05***	**< 0.05***
Ammonia (mg/L)	20.7±1.71	19.4±1.71		20.9±1.88	17.2±1.88		
Bicarbonate (mmol/L)	1.45±0.414	0.714±0.414		1.03±0.291	0.830±0.291		
Calcium (mmol/24hr)	2.28±0.323	1.83±0.323		3.75±0.439	2.65±0.439		
Citrate (mmol/24hr)	2.60±0.246	2.90±0.246		2.29±0.392	3.17±0.392		
*Chloride (mmol/24hr)	139±9.16	84.6±9.16	**< 0.05***	135±13.1	83.1±13.1	**< 0.05***	
Creatinine (mmol/24hr)	13.8±1.01	12.7±1.01		15.6±1.46	13.5±1.46		
Magnesium (mmol/24hr)	2.29±0.267	1.98±0.267		3.13±0.355	2.11±0.355	**< 0.05***	
Oxalate (mmol/24hr)	0.329± 0.025	0.289±0.025		0.343±0.021	0.303±0.021		
Phosphate (mmol/24hr)	23.4±2.54	16.6±2.54		27.5±0.187	20.7±0.187		
Potassium (mmol/24hr)	44.1±5.41	29.7±5.41		60.3±6.87	39.5±6.87		
*Sodium (mmol/24hr)	117±12.9	40.1±12.9	**< 0.05***	109±11.2	38.2±11.2	**< 0.05***	
Sulphate (mmol/24hr)	18.8±2.04	15.6±2.04		18.1±1.52	16.1±1.52		
*Uric acid (mmol/24hr)	3.06±0.194	2.46±0.194	**< 0.05***	3.31±0.237	2.79±0.237		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.055	0.263±0.055	**< 0.05***	0.481±0.0635	0.321±0.0635		
*BONN-Risk-Index (BRI)	1.02±0.184	0.596±0.184		1.69±0.272	1.48±0.272		**< 0.05***
Tiselius Risk Index (TRI)	285±34.9	251±34.9		350±36.9	292±36.9		
*RS Brushite	0.547±0.212	0.323±0.212		1.51±0.342	0.384±0.342	**< 0.05***	
*RS CaOx	2.34±0.712	2.56±0.712		5.87±0.569	2.63±0.569	**< 0.05***	
*RS Uric acid	1.87±0.228	1.68±0.228		2.18±0.374	1.41±0.374	**< 0.05***	
CaOx MSL (mmol/L)	90±11.8	60±11.8		75±12.8	75±12.8		

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.8: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n =19) subjects at day 5 of protocol H (high NaCl). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	High NaCl	P	Baseline	High NaCl	p	High NaCl p
pH	6.13±0.112	6.25±0.112		6.12± 0.099	6.29±0.099		
*Volume (mL/24hr)	1328±165	2026±165	**< 0.05***	1322±100	1523±100		**< 0.05***
Ammonia (mg/L)	20.7±1.91	22.5±1.91		20.9±2.39	21.6±2.39		
Bicarbonate (mmol/L)	1.45±0.432	1.31±0.432		1.03±0.451	1.71±0.451		
*Calcium (mmol/24hr)	2.28±0.362	2.79±0.362		3.75±0.524	4.57±0.524		**< 0.05***
*Citrate (mmol/24hr)	2.60±0.209	2.61±0.209		2.29±0.428	3.43±0.428		**< 0.05***
*Chloride (mmol/24hr)	139±16.7	237±16.7	**< 0.05***	135±18.8	221±18.8	**< 0.05***	
Creatinine (mmol/24hr)	13.8±1.01	14.7±1.01		15.6±1.59	16.1±1.59		
*Magnesium (mmol/24hr)	2.29±0.305	2.16±0.305		3.13±0.362	3.21±0.362		**< 0.05***
Oxalate (mmol/24hr)	0.329±0.025	0.292±0.025		0.343±0.0182	0.303±0.0182		
Phosphate (mmol/24hr)	23.4±2.48	24.0±2.48		27.5±2.96	31.2±2.96		
Potassium (mmol/24hr)	44.1±5.14	39.9±5.14		60.3±7.31	51.1±7.31		
*Sodium (mmol/24hr)	117±29.7	281±29.7	**< 0.05***	109±25.5	230±25.5	**< 0.05***	
*Sulphate (mmol/24hr)	18.8±1.98	20.0± 1.98		18.1±1.92	24.0±1.92	**< 0.05***	
Uric acid (mmol/24hr)	3.06±0.227	3.36±0.227		3.31±0.329	3.83±0.329		
Free Calcium (Ca^{2+}, mmol)	0.402±0.662	0.364±0.662		0.481±0.0912	0.563±0.0912		
*BONN-Risk-Index (BRI)	1.02±0.198	0.779±0.498		1.69± 0.356	1.947±0.356		**< 0.05***
Tiselius Risk Index (TRI)	285±34.3	251±34.3		350±41.1	308±41.1		
RS Brushite	0.547±0.142	0.615±0.142		1.51±0.358	0.932±0.358		
RS CaOx	2.34±0.363	2.68±0.363		5.87±0.595	4.556±0.595		
RS Uric acid	1.87±0.372	1.45±0.372		2.18±0.380	1.957±0.380		
CaOx MSL (mmol/L)	90±11.4	120±11.4		75±9.72	75±9.72		**< 0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.9: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 7 of protocol H (high NaCl). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	High NaCl	P	Baseline	High NaCl	p	High NaCl p
pH	6.13±0.105	6.35±0.105		6.12±0.108	6.37±0.108		
*Volume (mL/24hr)	1328±147	2038±147	< 0.05*	1322±118	1809±118	<0.05*	
Ammonia (mg/L)	20.7±3.14	22.7±3.14		20.9±2.75	20.1±2.75		
Bicarbonate (mmol/L)	1.45±0.595	1.59±0.595		1.03±0.297	1.09±0.297		
*Calcium (mmol/24hr)	2.28±0.343	2.03±0.343		3.75±0.461	4.86±0.461		<0.05*
Citrate (mmol/24hr)	2.60±0.278	3.09±0.278		2.29±0.422	3.04±0.422		
Chloride (mmol/24hr)	139±14.3	163±14.3		135±14.4	159±14.4		
Creatinine (mmol/24hr)	13.8±0.985	12.2±0.985		15.6±1.42	12.8±1.42		
*Magnesium (mmol/24hr)	2.29±0.276	1.86±0.276		3.13±0.353	2.46±0.353		<0.05*
*Oxalate (mmol/24hr)	0.329±0.0262	0.278±0.0262		0.343±0.021	0.276±0.021	<0.05*	
*Phosphate (mmol/24hr)	23.4±2.39	15.6±2.39	< 0.05*	27.5±2.16	17.8±2.16	<0.05*	
*Potassium (mmol/24hr)	44.1±5.08	31.7±5.08		60.3±6.88	37.0±6.88	<0.05*	
Sodium (mmol/24hr)	117±17.4	112±17.4		109±14.7	87.7±14.7		
Sulphate (mmol/24hr)	18.8±2.02	13.6±2.02		18.1±1.53	15.6±1.53		
*Uric acid (mmol/24hr)	3.06±0.216	2.44±0.216	< 0.05*	3.31±0.222	2.79±0.222		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.0482	0.228±0.0482	< 0.05*	0.481±0.0671	0.347±0.0671		
*BONN-Risk-Index (BRI)	1.02±0.168	0.699±0.168		1.69±0.277	1.47±0.277		<0.05*
Tiselius Risk Index (TRI)	285±33.4	267±33.4		350±39.3	301±39.3		
*RS Brushite	0.547±0.118	0.302±0.118		1.51±0.0238	0.371±0.0238	<0.05*	
*RS CaOx	2.34±0.326	1.96±0.326		5.87±0.556	2.66±0.556	<0.05*	
*RS Uric acid	1.87±0.302	0.850±0.302	< 0.05*	2.18±0.365	0.669±0.365	<0.05*	<0.05*
CaOx MSL (mmol/L)	90±11.3	120±11.3		75±11.1	90±11.1		<0.05*

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.10: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 5 of protocol C (sodium bicarbonate, $NaHCO_3$). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	$NaHCO_3$	P	Baseline	$NaHCO_3$	p	$NaHCO_3$ p
*pH	6.13±0.105	6.87±0.105	**<0.05***	6.12±0.098	6.81±0.098	**<0.05***	
Volume (mL/24hr)	1328±166	1757±166		1322±123	1547±123		
Ammonia (mg/L)	20.7±4.39	25.4±4.39		20.9±2.54	18.1±2.54		
*Bicarbonate (mmol/L)	1.45±0.625	2.76±0.625		1.03±0.548	2.52±0.548	**<0.05***	
*Calcium (mmol/24hr)	2.28±0.329	1.81±0.329		3.75±0.543	3.66±0.543		**<0.05***
Citrate (mmol/24hr)	2.60±0.273	3.10±0.273		2.29±0.418	3.10±0.418		
Chloride (mmol/24hr)	139±12.3	126±12.3		135±17.5	168±17.5		
Creatinine (mmol/24hr)	13.8±0.995	12.6±0.995		15.6±1.51	14.4±1.51		
*Magnesium (mmol/24hr)	2.29±0.251	1.78±0.251		3.13±0.413	2.63±0.413		**<0.05***
*Oxalate (mmol/24hr)	0.329±0.0271	0.323±0.0271		0.343±0.0202	0.288±0.0202	**<0.05***	
Phosphate (mmol/24hr)	23.4±2.54	20.3±2.54		27.5±2.77	25.4±2.77		
*Potassium (mmol/24hr)	44.1±5.46	35.7±5.46		60.3±8.28	61.5±8.28		**<0.05***
Sodium (mmol/24hr)	117±24.2	139±24.2		109±24.3	197±24.3		
Sulphate (mmol/24hr)	18.8±2.27	18.4±2.27		18.1±1.75	21.0±1.75		
Uric acid (mmol/24hr)	3.06±0.251	2.97±0.251		3.31±0.275	3.59±0.275		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.0454	0.152±0.0454	**<0.05***	0.481±0.0683	0.312±0.0683	**<0.05***	**<0.05***
*BONN-Risk-Index (BRI)	1.02±0.174	0.499±0.174	**<0.05***	1.69±0.248	1.084±0.248	**<0.05***	**<0.05***
Tiselius Risk Index (TRI)	285±37.1	256±37.1		350±39.2	285±39.2		
RS Brushite	0.547±0.159	0.585±0.159		1.51±0.372	1.04±0.372		
*RS CaOx	2.34±0.371	2.207±0.371		5.87±0.649	0.429±0.649	**<0.05***	
*RS Uric acid	1.87±0.298	0.561±0.298	**<0.05***	2.18±0.361	1.09±0.361	**<0.05***	
CaOx MSL (mmol/L)	90±13.2	120±13.2		75±11.4	75±11.4		**<0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.11: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 7 of protocol C (sodium bicarbonate, $NaHCO_3$). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	$NaHCO_3$	P	Baseline	$NaHCO_3$	p	$NaHCO_3$ p
*pH	6.13±0.091	6.87±0.091	<0.05*	6.12±0.097	6.76±0.097	<0.05*	
*Volume (mL/24hr)	1328±172	2008±172	<0.05*	1322±131	1914±131	<0.05*	
Ammonia (mg/L)	20.7±3.88	20.4±3.88		20.9±3.93	19.6±3.93		
Bicarbonate (mmol/L)	1.45±0.473	1.67±0.473		1.03±0.566	2.01±0.566		
*Calcium (mmol/24hr)	2.28±0.368	1.15±0.368	<0.05*	3.75±0.435	2.08±0.435	<0.05*	
*Citrate (mmol/24hr)	2.60±0.305	3.91±0.305	<0.05*	2.29±0.441	3.71±0.441	<0.05*	
*Chloride (mmol/24hr)	139 ±10.2	95.8±10.2	<0.05*	135±12.8	70.7±12.8		<0.05*
Creatinine (mmol/24hr)	13.8±0.952	12.8±0.952		15.6±1.42	12.7±1.42		
Magnesium (mmol/24hr)	2.29±0.276	2.11±0.276		3.13±0.362	2.15±0.362		
Oxalate (mmol/24hr)	0.329±0.0444	0.366±0.0444		0.343±0.0184	0.261±0.0184		
*Phosphate (mmol/24hr)	23.4±2.26	14.9±2.26	<0.05*	27.5±2.27	19.6±2.27		<0.05*
Potassium (mmol/24hr)	44.1±5.51	35.2±5.51		60.3±6.79	37.5±6.79		
Sodium (mmol/24hr)	117±18.9	82.0±18.9		109±12.6	52.7±12.6		
Sulphate (mmol/24hr)	18.8±2.06	13.3±2.06		18.1±1.81	14.6±1.81		
Uric acid (mmol/24hr)	3.06±0.192	2.54±0.192		3.31±0.226	2.66±0.226		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.462	0.134±0.0462	<0.05*	0.481±0.0566	0.163±0.0566	<0.05*	
*BONN-Risk-Index (BRI)	1.02±0.172	0.430±0.172	<0.05*	1.69±0.246	0.812±0.246	<0.05*	
Tiselius Risk Index (TRI)	285±37.6	241±37.6		350±40.5	257±40.5		
*RS Brushite	0.547±0.125	0.368±0.125		1.51±0.346	0.497±0.346	<0.05*	
*RS CaOx	2.34±0.442	2.24±0.442		5.87±0.589	2.182±0.589	<0.05*	
*RS Uric acid	1.87±0.387	0.690±0.387	<0.05*	2.18±0.359	0.324±0.359	<0.05*	
CaOx MSL (mmol/L)	90±11.2	105±11.2		75±13.2	90±13.2		

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.12: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 5 of protocol D (sodium citrate). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	Citrate	P	Baseline	Citrate	p	Citrate p
*pH	6.13±0.122	6.99±0.122	<0.05*	6.12±0.101	6.99±0.101	<0.05*	
*Volume (mL/24hr)	1328±174	1637±174		1322±115	1655±115	<0.05*	
Ammonia (mg/L)	20.7±1.62	16.7±1.62		20.9±4.351	29.4±4.351		
*Bicarbonate (mmol/L)	1.45±0.577	2.79±0.577		1.03±0.543	2.53±0.543	<0.05*	
Calcium (mmol/24hr)	2.28±0.329	1.79±0.329		3.75±0.485	2.53±0.485		
Citrate (mmol/24hr)	2.60±0.322	3.15±0.322		2.29±0.397	2.89±0.397		
Chloride (mmol/24hr)	139±12.8	126±12.8		135±15.8	115±15.8		
Creatinine (mmol/24hr)	13.8± 0.910	12.6±0.910		15.6±1.51	13.2±1.51		
Magnesium (mmol/24hr)	2.29± 0.258	1.85±0.258		3.13±0.436	2.52±0.436		
Oxalate (mmol/24hr)	0.329±0.335	0.343±0.335		0.343±0.0214	0.301±0.0214		
*Phosphate (mmol/24hr)	23.4±2.27	19.4±2.27		27.5±3.59	28.5±3.59		<0.05*
*Potassium (mmol/24hr)	44.1±5.09	16.7±5.09	<0.05*	60.3±7.69	46.9±7.69		<0.05*
Sodium (mmol/24hr)	117± 27.5	168±27.5		109±21.2	168±21.2		
Sulphate (mmol/24hr)	18.8±2.003	15.6±2.003		18.1±2.03	18.5±2.03		
Uric acid (mmol/24hr)	3.06± 0.210	2.81±0.210		3.31± 0.315	3.33±0.315		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.0511	0.153±0.0511	<0.05*	0.481±0.0547	0.148±0.0547	<0.05*	
*BONN-Risk-Index (BRI)	1.02±0.177	0.369±0.177	<0.05*	1.69±0.291	0.793±0.291	<0.05*	
Tiselius Risk Index (TRI)	285±37.5	289±37.5		350±38.5	292±38.5		
*RS Brushite	0.547±0.134	0.534±0.134		1.51±0.359	0.912±0.359		<0.05*
*RS CaOx	2.34±0.377	2.153±0.377		5.87±0.549	2.36±0.549	<0.05*	
*RS Uric acid	1.86±0.312	0.430±0.312	<0.05*	2.18±0.353	0.227±0.353	<0.05*	
CaOx MSL (mmol/L)	90±14.2	105±14.2		75±14.2	90±14.2		

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.13: Mean urinary parameters (± SE) of samples from black (n = 20) and white (n = 19) subjects at day 7 of protocol D (sodium citrate). A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

	Blacks			Whites			B vs. W
Parameter	Baseline	Citrate	P	Baseline	Citrate	p	Citrate p
*pH	6.13±0.104	7.114±0.104	**<0.05***	6.12 ±0.109	7.03±0.109	**<0.05***	
*Volume (mL/24hr)	1328±189	1834±189	**<0.05***	1322 ±151	1964±151	**<0.05***	
*Ammonia (mg/L)	20.7±3.42	20.3±3.42		20.9±4.892	32.2±4.892		**<0.05***
*Bicarbonate (mmol/L)	1.45±0.425	1.32±0.425		1.03±0.435	2.15±0.435	**<0.05***	
*Calcium (mmol/24hr)	2.28±0.315	1.37±0.615	**<0.05***	3.75±0.456	2.32±0.456	**<0.05***	
*Citrate (mmol/24hr)	2.60±0.277	3.42±0.277	**<0.05***	2.29±0.446	4.06±0.446	**<0.05***	
*Chloride (mmol/24hr)	139±10.7	93.8±10.7	**<0.05***	135±15.8	115±15.8		**<0.05***
Creatinine (mmol/24hr)	13.8±0.887	12.3±0.887		15.6±1.39	12.6±1.39		
Magnesium (mmol/24hr)	2.29± 0.267	2.06±0.267		3.13±0.382	2.56±0.382		
*Oxalate (mmol/24hr)	0.329±0.032	0.331±0.032		0.343±0.0199	0.266±0.0199	**<0.05***	
*Phosphate (mmol/24hr)	23.4±2.14	15.5±2.14	**<0.05***	27.5±2.13	16.9±2.13		
*Potassium (mmol/24hr)	44.1±5.35	28.7±5.35	**<0.05***	60.3±6.96	42.6±6.96		
Sodium (mmol/24hr)	117±18.9	90.5±18.9		109±19.3	163±19.3		
Sulphate (mmol/24hr)	18.8±1.98	14.5±1.98		18.1±1.89	15.2±1.89		
*Uric acid (mmol/24hr)	3.06±0.189	2.54±0.189	**<0.05***	3.31±0.233	2.82±0.233		
*Free Calcium (Ca^{2+}, mmol)	0.402±0.0494	0.159±0.0494	**<0.05***	0.481±0.0538	0.106±0.0538	**<0.05***	
*BONN-Risk-Index (BRI)	1.02±0.211	0.553±0.211	**<0.05***	1.69±0.194	0.323±0.194	**<0.05***	
*Tiselius Risk Index (TRI)	285±38.9	262±38.9		350±34.5	223±34.5	**<0.05***	
RS Brushite	0.547±0.114	0.374±0.114		1.51±0.366	0.697±0.366		
*RS CaOx	2.34±0.387	1.26±0.387	**<0.05***	5.87±0.549	1.85±0.549	**<0.05***	
*RS Uric acid	1.87±0.279	0.271±0.279	**<0.05***	2.18 ±0.425	0.601±0.425	**<0.05***	
*CaOx MSL (mmol/L)	90±13.7	120±13.7		75±11.2	90±11.2	**<0.05***	**<0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). RS: Relative Supersaturation.*

Table 6.14: A summary of common and different physicochemical changes in samples from black and white subjects after each protocol.

Protocol	Common changes in B & W	Differences (independent of baseline)	
		B	W
Low NaCl (L)			
Day 5 (L5) (Table 6.6)	—	—	—
Day 7 (L7) (Table 6.7)	Volume ↑, Chloride ↓, Sodium ↓	Uric acid ↓, Ca^{2+} ↓	Magnesium ↓, RS brushite ↓, RS CaOx ↓, RS Uric acid↓
High NaCl (H)			
Day 5 (H5) (Table 6.8)	Chloride ↑, Sodium ↑	Volume ↑	Sulphate ↑
Day 7 (H7) (Table 6.9)	Volume ↑, Phosphate ↓, RS Uric acid ↓	Uric acid ↓, Ca^{2+} ↓	Oxalate↓, Potassium ↓, RS brushite ↓, RS CaOx ↓
$NaHCO_3$ (C)			
Day 5 (C5) (Table 6.10)	pH ↑, Ca^{2+} ↓, BRI ↓, RS Uric acid ↓		Bicarbonate ↑, Oxalate ↓, RS CaOx ↓
Day 7 (C7) (Table 6.11)	pH ↑, Volume↑, Calcium ↓, Citrate ↑, Ca^{2+} ↓, BRI ↓, RS Uric acid ↓	Chloride ↓, Phosphate ↓	RS brushite ↓, RS CaOx ↓
Na-Citrate (D)			
Day 5 (D5) (Table 6.12)	pH ↑, Ca^{2+} ↓, BRI ↓, RS Uric acid ↓	Potassium ↓,	Volume↑, Bicarbonate ↑, RS CaOx ↓
Day 7 (D7) (Table 6.13)	pH ↑, Volume ↑, Calcium ↓, Citrate ↑, Ca^{2+} ↓, BRI ↓, RS CaOx ↓, RS Uric acid ↓	Chloride ↓, Phosphate ↓, Potassium ↓, Uric acid ↓	Bicarbonate ↑, Oxalate ↑, TRI ↓, MSL ↑

Comment

In this study, baseline urine samples from black subjects had significantly lower calcium concentrations, lower BRI, and lower RS of brushite and RS of CaOx compared to those from their white compatriots (Table 6.5). All these parameters are important risk factors for kidney stone formation. The significant differences in these parameters are in accordance with the low incidence of kidney stone formation in the black South African race group. In particular, the lower RS of CaOx in black subjects is highly indicative of their lower stone incidence.

The baseline urine composition values in this study (Table 6.5) do not agree with those reported in the study on macromolecules in Chapter 3 (Table 3.1), with the exception of RS of CaOx which was found to be significantly lower in black subjects in both studies. The discrepancies in the other compositional values are likely to be due to the higher statistical power of the present study which involved 20 subjects, as compared to only 10 subjects in the other.

It is of some interest to consider the changes which commonly occurred in both race groups after each of the protocols (Tables 6.14). Regarding the sodium chloride protocols, it is not surprising that urinary sodium and chloride decreased (relative to baseline values) after the low NaCl protocol and increased after the high NaCl protocol. On the other hand, the decreases in urinary phosphate and RS of uric acid are unexpected as they have not been previously reported. The physiological mechanisms giving rise to these two effects are not apparent.

Regarding the sodium bicarbonate protocols, pH increased in both groups. This was accompanied by a concomitant decrease in ionized calcium. This effect can be attributed to complexation of free calcium with citrate (Lemann et al. 1989). Indeed, a recent study by Rodgers et al. (2005) identified a new calcium-citrate-phosphate complex which forms at elevated pH levels. The decrease in the BRI is a direct consequence of the decrease in ionized calcium. Of great interest in the present study is the observation of a decrease in total urinary calcium in both groups after administration of sodium bicarbonate. This has not been previously reported. In fact, to the contrary, Lemann et al. (1989) specifically drew attention to the fact that this effect was achieved by potassium bicarbonate but not by sodium

bicarbonate. Another interesting effect is the increase in urinary citrate. This observation has been previously reported by Lemann et al. (1989) and might be attributable to the expected rise in pH and the decrease (but not significant) in uric acid after administration of sodium bicarbonate (Lemann et al.).

The common changes achieved after sodium citrate protocols (increase in urinary pH, decrease in ionized calcium and BRI) occurred for the same reasons as explained above for sodium bicarbonate. Furthermore, while the increase in urinary citrate is expected, the decrease in total urinary calcium has not been previously observed after administration of sodium citrate (Sakhaee 1983; Preminger 1988). However, this might be due to the fact that the preparation which was used in the present study contained tartrate and bicarbonate in addition to sodium citrate.

It is noted that all the protocols (except low NaCl) induced a decrease in RS of uric acid. As mentioned above, the physiological mechanism that gives rise to this effect after the high NaCl protocol is unknown. However, with respect to the sodium bicarbonate and sodium citrate protocols, the effect is readily attributed to the increase in urinary pH.

Finally, the failure of the high NaCl protocol to induce statistically significant increases in urinary calcium is surprising since this effect has been widely reported (Kleeman et al. 1964; Rao et al. 1985; Sakhaee et al. 1993; Ginty et al. 1998; Ho et al. 2001; Massey 2005; Wigerts et al. 2005). However, King et al. (1964), reported no significant increase in urinary calcium following administration of 8 g of NaCl for 6 days. This was attributable to the fact that the reserves of labile bone sodium gradually become depleted and repleted in a cycle form. In such cases, repletion requires a longer period to recover from the longer period of depletion (Neuman and Neuman 1958). Nevertheless, despite the absence of a statistically significant increase in urinary calcium, it is noted that after 7 days on the high NaCl diet, urinary calcium increased by 29 % in whites, but decreased in blacks by 10.9 % (Table 6.9). There is thus a hint of different handling mechanisms in the two race groups.

Of even greater interest than the common changes are the effects which occurred in one race group but not the other in each of the protocols. There were fewer changes in the urine chemistries of black subjects than in those of white subjects. All of the changes in both groups were favourable, i.e. they reduced the physiochemical risk of calcium oxalate

crystallization. The decreases in RS of CaOx, RS of uric acid and RS of brushite after the various protocols in white subjects have not been previously reported.

However, irrespective of whether the effects have been previously reported or not and irrespective of the process giving rise to them, the observation of different responses in the two groups, despite identical conditions being imposed, indicates that handling of the protocols in the two groups proceeds via different mechanisms.

6.4.2 Crystallization Experiments

CaOx Metastable Limit

Although the mean MSL values and standard errors (SE) of samples from both black 9 (n = 20) and white (n = 19) subjects on the various protocols have already been presented in Tables 6.5 -6.13, they have been consolidated into a single table (6.15) for ease of reference. Only the statistically significant comparisons are shown. Corresponding histograms are given in Figure 6.4. Qualitatively, urine samples from black subjects have relatively higher CaOx metastable limits than those from white subjects at baseline and after each protocol. However, statistically significant higher metastable limits in blacks were recorded after the H5, H7, C5 and D7 protocols.

Table 6.15: Mean Metastable Limit (mmol/L), and standard errors (SE) of urine from black (B, n = 20) and white (W, n = 19) subjects at baseline and at Day 5 and Day 7 of each salt.

Protocol	B ± SE	B: Baseline vs. Protocol (p-values)	W ± SE	W: Baseline vs. Protocol (p-values)	B vs. W p-values
Baseline	90 ± 11		75 ± 11		
Low NaCl, day 5 (L5)	60 ± 10		75 ± 10		
Low NaCl, day 7 (L7)	90 ± 14		90 ± 14		
High NaCl, day 5 (H5)	120 ± 11	**< 0.05***	75 ± 11		**< 0.05***
High NaCl day 7 (H7)	120 ± 12	**< 0.05***	90 ± 12		**< 0.05***
$NaHCO_3$, day 5 (C5)	120 ± 14	**< 0.05***	75 ± 14		**< 0.05***
$NaHCO_3$, day 7 (C7)	105 ± 14		90 ± 14		
Na-Citrate, day 5 (D5)	105 ± 17		90 ± 17		
Na-Citrate, day 7 (D7)	120 ± 14	**< 0.05***	90 ± 14		**< 0.05***

** Difference is statistically significant: $p \leq 0.05$, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

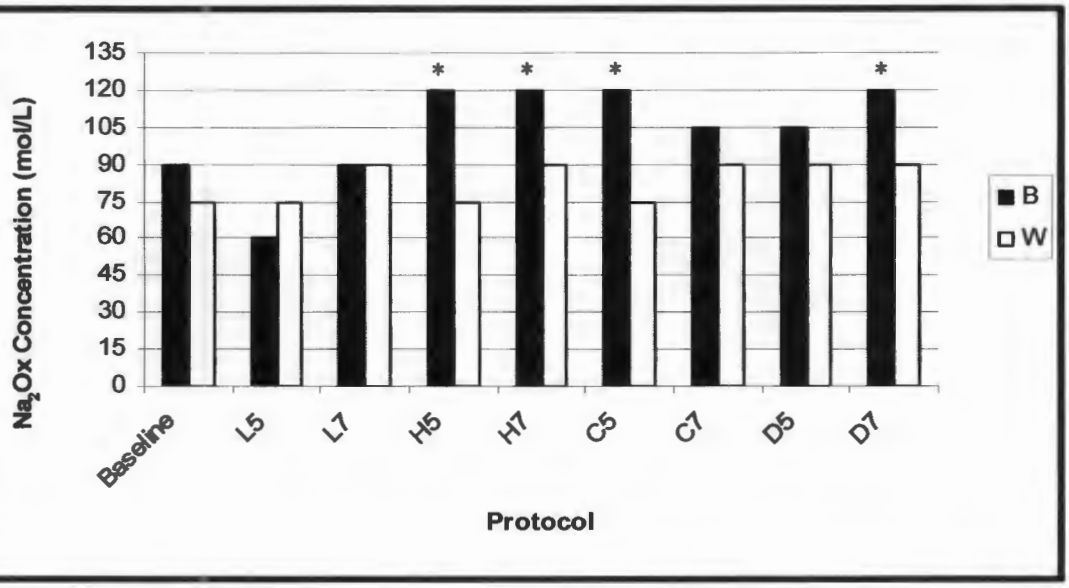


Figure 6.4: Mean MSL histogram of urine samples from black (B) and white (W) subjects at baseline, day 5 and day 7 of each protocol.

* : Difference is statistically significant relative to the corresponding W protocol.

Comment

The higher MSL of urine samples from black subjects compared to those from white subjects is in agreement with that reported in Chapter 3 and supports the notion that crystallization of CaOx in the urine of the former group is more difficult to achieve. Interestingly, administration of high NaCl, high sodium bicarbonate and high sodium citrate induced a significant increase in the MSL in blacks whereas no change was observed in whites. This provides further support for the hypothesis that different handling mechanisms of lithogenic dietary challenges exist in the two groups.

Particle Number

Tables 6.16 and 6.17 represent the particle number (/500 μL) of urine samples from black (n = 20) and white (n = 19) subjects, respectively at baseline and at day 5 and day 7 of each protocol. The corresponding histogram is shown in Figure 6. 5. Individual values are presented in Appendix 4, Tables 4.3 and 4.4.

Table 6.16: Mean particle number (/500 μL), standard errors and p-values in urine samples from black subjects (n = 20) at baseline and at Day 5 and Day 7 of each protocol. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle Number ± S. E.	% Increase wrt Baseline	% Decrease wrt Baseline	p-Value wrt Baseline	B vs. W p-values
Baseline	24100 ± 3218				
L5	28889 ± 3283	16.57		0.3109	
L7	27747 ± 2737	13.14		0.4244	**< 0.05***
H5	23037 ± 3119		4.41	0.8158	
H7	22183 ± 2822		7.95	0.6618	
C5	20160 ± 3182		16.35	0.3943	
C7	22911 ± 3196		4.93	0.8075	**< 0.05***
D5	24278 ± 4159	0.73		0.9770	
D7	16998 ± 2792		29.47	0.1307	

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

Table 6.17: Mean particle number (/500 µL), standard errors and p-values in urine samples from white subjects (n = 19) at baseline and at Day 5 and Day 7 of each protocol. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle Number ± S. E.	% Increase wrt Baseline	% Decrease wrt Baseline	p-Value wrt Baseline	B vs. W p-values
Baseline	25137 ± 3218				
L5	26101 ± 3283	3.69		0.8368	
L7	17126 ± 2737		31.87	0.052	**< 0.05***
H5	26999 ± 3119	6.89		0.6843	
H7	20898 ± 2822		16.86	0.3325	
C5	25910 ± 3182	2.98		0.8656	
C7	16472 ± 3196		34.47	0.0508	**< 0.05***
D5	23445 ± 4159		6.73	0.6915	
D7	20504 ± 7292		18.43	0.2512	

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

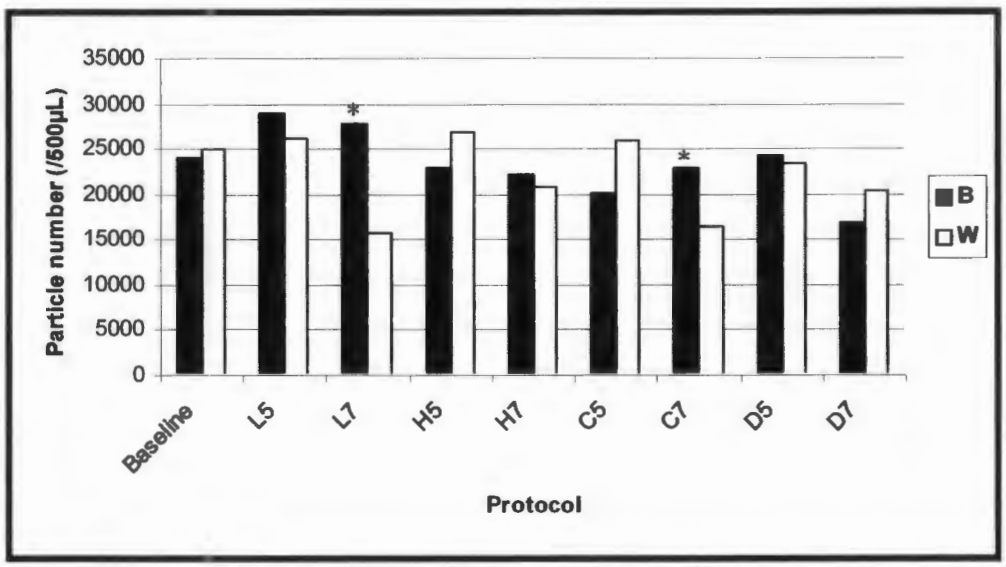


Figure 6.5: Mean particle number (/500µL in urine samples from black (B) and white (W) subjects at baseline, day 5 and day 7 of each protocol.

* : Difference is statistically significant relative to the corresponding W protocol.

B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.

There was no significant difference in particle numbers in both race groups after the ingestion of each protocol. However, in white subjects, decreases which tended towards significance were observed after the low NaCl diet on day 7 (L7, Table 6.16) and after $NaHCO_3$ diet on day 7 (C7, Table 6.16). Comparison of the two groups themselves showed a significantly higher number of particles in black subjects on day 7 of the low NaCl diet (L7).

Comment

Although the physiological explanation for the above observations is not immediately apparent, the importance of these observations is that differences between the groups, irrespective of their reasons, do indeed exist.

Particle Volume

Table 6.18 and 6.19 show the mean particle volume of urine samples from black (n = 20) and white (n = 19) subjects, respectively at baseline and at day 5 and day 7 of each protocol. The corresponding histogram is shown in Figure 6. 6. The individual data are shown in Appendix 4, Tables 4.5 and 4.6.

Table 6.18: Mean particle volume (x 10^6 μm^3/500 µL), standard errors and p-values in urine samples from black subjects (n = 20) at baseline and at Day 5 and Day 7 of each protocol. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle volume ± S. E. (x 10^6 μm^3/500 µL)	% Increase wrt baseline	% Decrease wrt baseline	p-Value wrt baseline (> 0.05)	B vs. W p-values
Baseline	1.34 ± 0.213				**< 0.05***
L5	4.26 ± 0.339	68.47			**< 0.05***
L7	1.48 ± 0.117	9.32			
H5	1.30 ± 0.113		2.83		**< 0.05***
H7	1.60 ± 0.187		16.13		
C5	1.682 ± 0.234		20.21		**< 0.05***
C7	0.834 ± 0.091	37.85			
D5	0.661 ± 0.212	50.75			**< 0.05***
D7	1.866 ± 0.321	28.08			

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

Table 6.19: Mean particle volume, standard errors and p-values of urine samples from white subjects (n = 19) at baseline and at Day 5 and Day 7 of each protocol. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle volume ± S. E. (x 10^6 μm^3/500 µL)	% Increase wrt baseline	% Decrease wrt baseline	p-value wrt baseline (> 0.05)	B vs. W p-values
Baseline	2.19 ± 0.213				**< 0.05***
L5	2.18 ± 0.339		0.27		**< 0.05***
L7	1.46 ± 0.117		33.42		
H5	2.75 ± 0.113	20.16			**< 0.05***
H7	1.61 ± 0.187		26.40		
C5	3.12 ± 0.234	42.13			**< 0.05***
C7	1.25 ± 0.091		43.14		
D5	3.30 ± 0.212	33.59			**< 0.05***
D7	2.19 ± 0.321				

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

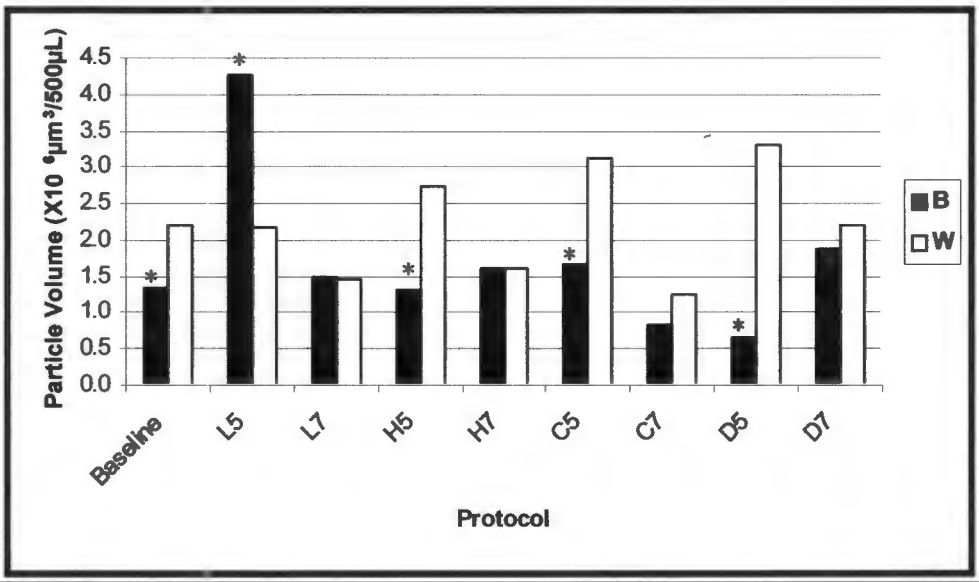


Figure 6.6: Mean particle volume in urine samples from both black (B) and white (W) subjects at baseline and at day 5 and day 7 of each protocol.

* : Difference is statistically significant relative to the corresponding W protocol.

** Difference is statistically significant: $p \leq 0.05$, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

There was no significant difference in the particle volume in both race groups after the ingestion of each protocol. However, several statistically significant differences were recorded when comparisons were made between the two ethnic groups themselves. Particle volume at baseline is lower in samples from black subjects than in those from white subjects (Figure 6.6). While this relationship was retained after protocols H5, C5, C7 and D5, it was reversed after protocol L5 (Figure 6.6).

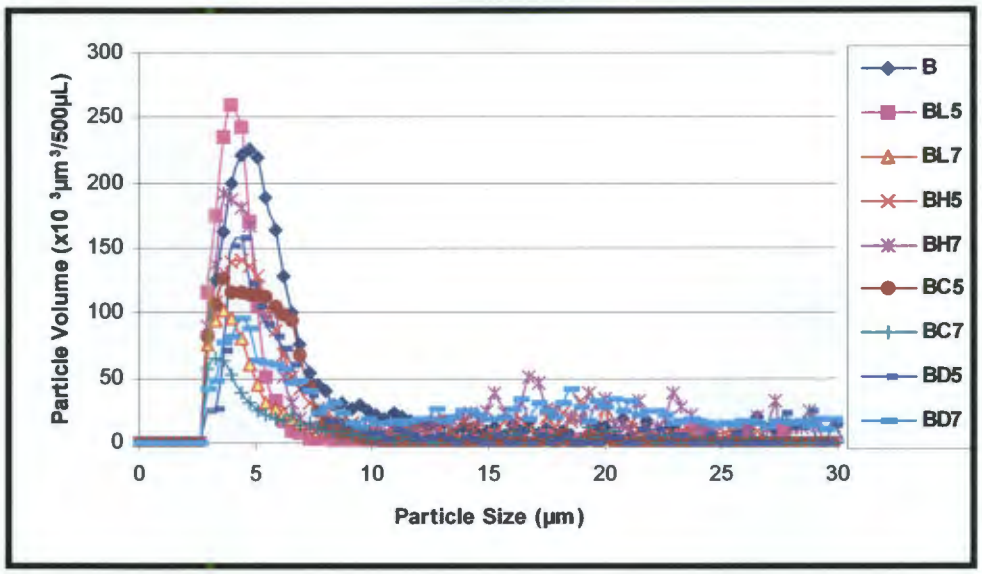
Comment

The higher total particle volume in white subjects (despite non significant differences in baseline particle numbers reported in Tables 6.16 and 6.17) is indicative of larger particles or a greater extent of aggregation (with occluded air and debris) or both. However, interpretation of the reversal of the trend after protocol L5 is not apparent at this stage.

Particle Volume-Size Distributions

The mean particle-size distribution plots in the urine of blacks (n = 20) and whites (n = 19) before and after each protocol are shown in Figure 6. 7 (a) and (b), respectively. Individual plots are in Appendix 4, Figures 4.1 and 4.2. The corresponding mean particle size values with p-values are presented in Tables 6.20 and 6.21.

The size of crystals induced in samples from white subjects at baseline and at day 5 and 7 of each protocol was significantly higher than those from black subjects. When inter-group comparisons were made, there were no significant changes in particle size after each protocol relative to the control. However, all the protocols resulted in a decrease in particle size in samples from black subjects relative to the baseline whereas an increase was observed in samples from white subjects.



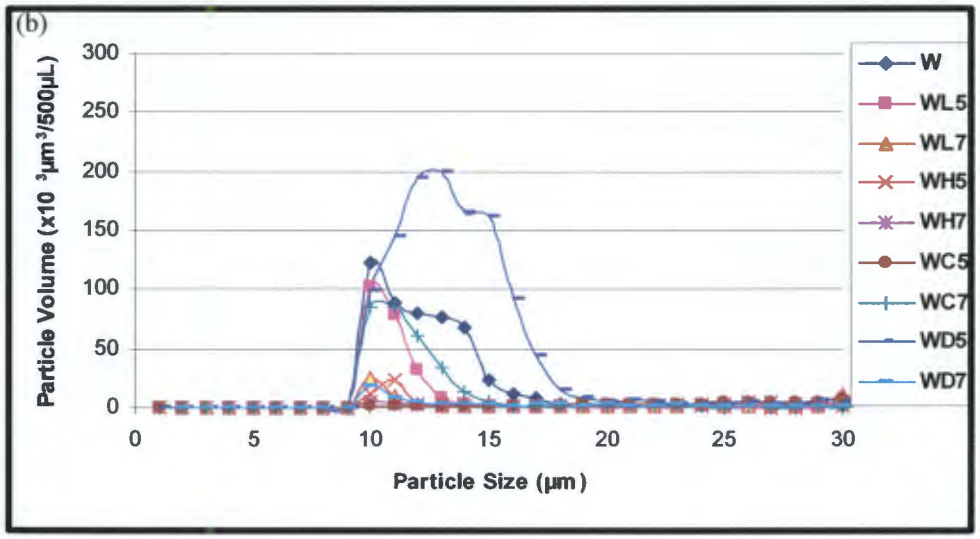


Figure 6.7: Mean particle volume-size distribution of urine samples from (a) black and (b) white at baseline and day 5 and day 7 of each protocol.

Table 6.20: Mean particle sizes, standard errors and p-values in urine samples from black subjects at baseline, and at Day 5 and Day 7 of each protocol. A p-value of ≤ 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle size (μm) ± S. E.	% Decrease wrt baseline	p-Value wrt baseline (p > 0.05)	B vs. W (p-values)
Baseline	4.53 ± 0.985			**< 0.05***
*L5	4.27 ± 1.03	5.74		**< 0.05***
*L7	4.09 ± 0.695	9.71		**< 0.05***
*H5	4.39 ± 0.844	3.09		**< 0.05***
*H7	4.13 ± 0.731	8.83		**< 0.05***
*C5	4.03 ± 1.14	11.0		**< 0.05***
*C7	3.93 ± 0.926	13.3		**< 0.05***
*D5	4.29 ± 1.67	5.29		**< 0.05***
*D7	4.28 ± 1.23	5.52		**< 0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

Table 6.21: Mean particle sizes, standard errors and p-values in urine samples from white subjects at baseline and at Day 5 and Day 7 of each protocol. A p-value of < 0.05 with a (*) is only shown where there is a statistically significant difference.

Protocol	Particle size (µm) ± S. E.	% Increase wrt baseline	p-Value wrt baseline ($p > 0.05$)	B vs. W (p -values)
*Baseline	9.89 ± 1.01			**< 0.05***
*L5	10.1 ± 1.05	2.08		**< 0.05***
*L7	10.3 ± 0.713	4.07		**< 0.05***
*H5	10.7 ± 0.866	7.39		**< 0.05***
*H7	9.99 ± 0.751	0.90		**< 0.05***
*C5	9.99 ± 1.16	0.90		**< 0.05***
*C7	10.7 ± 0.951	7.82		**< 0.05***
*D5	13.2 ± 1.72	25.1		**< 0.05***
*D7	10.1 ± 1.23	1.88		**< 0.05***

** Difference is statistically significant: $p \leq 0.05$, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

Comment

Particle volume-size distribution curves reveal a significantly high particle size in samples from whites compared to those from blacks after all the protocols. This suggests promotion of crystal growth or aggregation in the samples from white subjects. Notwithstanding the fact that the differences were non-significant, a decrease in particle size was observed in blacks whereas an inverse was shown in samples from white subjects after all the protocols. This demonstrates inhibition of crystal growth or aggregation in blacks following the administration of the protocols whereas promotion occurred in whites.

Scanning Electron Microscopy (SEM)

Figure 6.8 shows representative scanning electron micrographs of crystals precipitated after induction of CaOx crystallization in baseline and day 7 urine samples obtained from black male subjects. At baseline, relatively few single COM crystals and a few tiny aggregates of COD crystals were observed (B Ctrl). After the NaCl protocols the number of COM crystals increased dramatically. These effects were significantly reduced after sodium bicarbonate and sodium citrate ingestion.

Figure 6.9 shows representative scanning electron micrographs of crystals precipitated after induction of CaOx crystallization in baseline and day 7 urine samples obtained from white male subjects. At baseline, medium-sized COD crystals were precipitated while after the low NaCl protocol, only one tiny aggregate was observed. Ingestion of high NaCl resulted in a change in morphology from medium-sized COD crystals to medium-sized single and aggregated COM crystals. The number of COM crystals was significantly reduced after sodium bicarbonate. Nevertheless, several were still present. After ingestion of sodium citrate, very few single or aggregated crystals were observed.

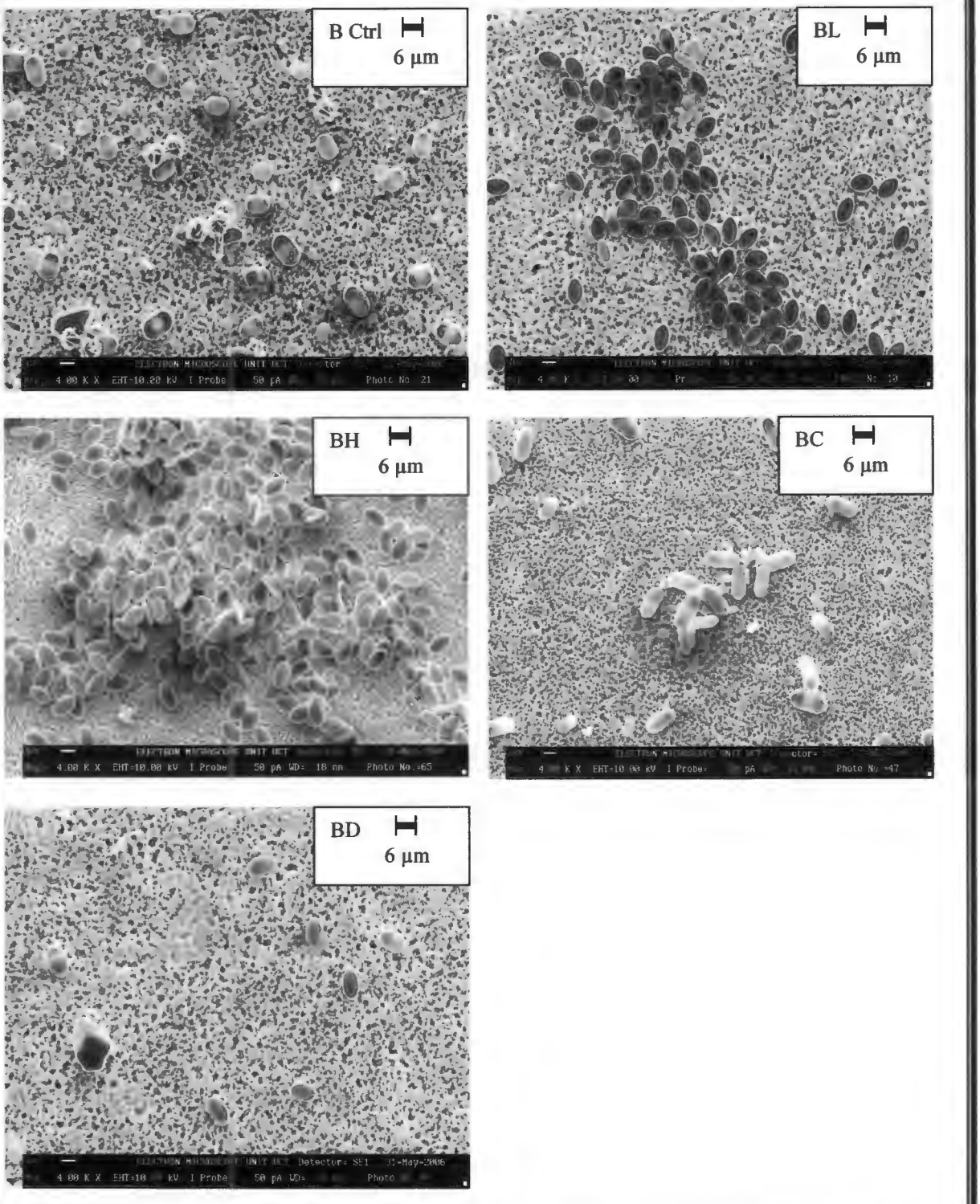


Figure 6.8: Scanning electron micrographs of crystals induced in urine samples from black (B) subjects at baseline and at day 7 of each protocol at 4 K magnification. B Ctrl (Baseline); BL (Low NaCl); BH (High NaCl); BC (Sodium Bicarbonate) and BD (Sodium citrate).

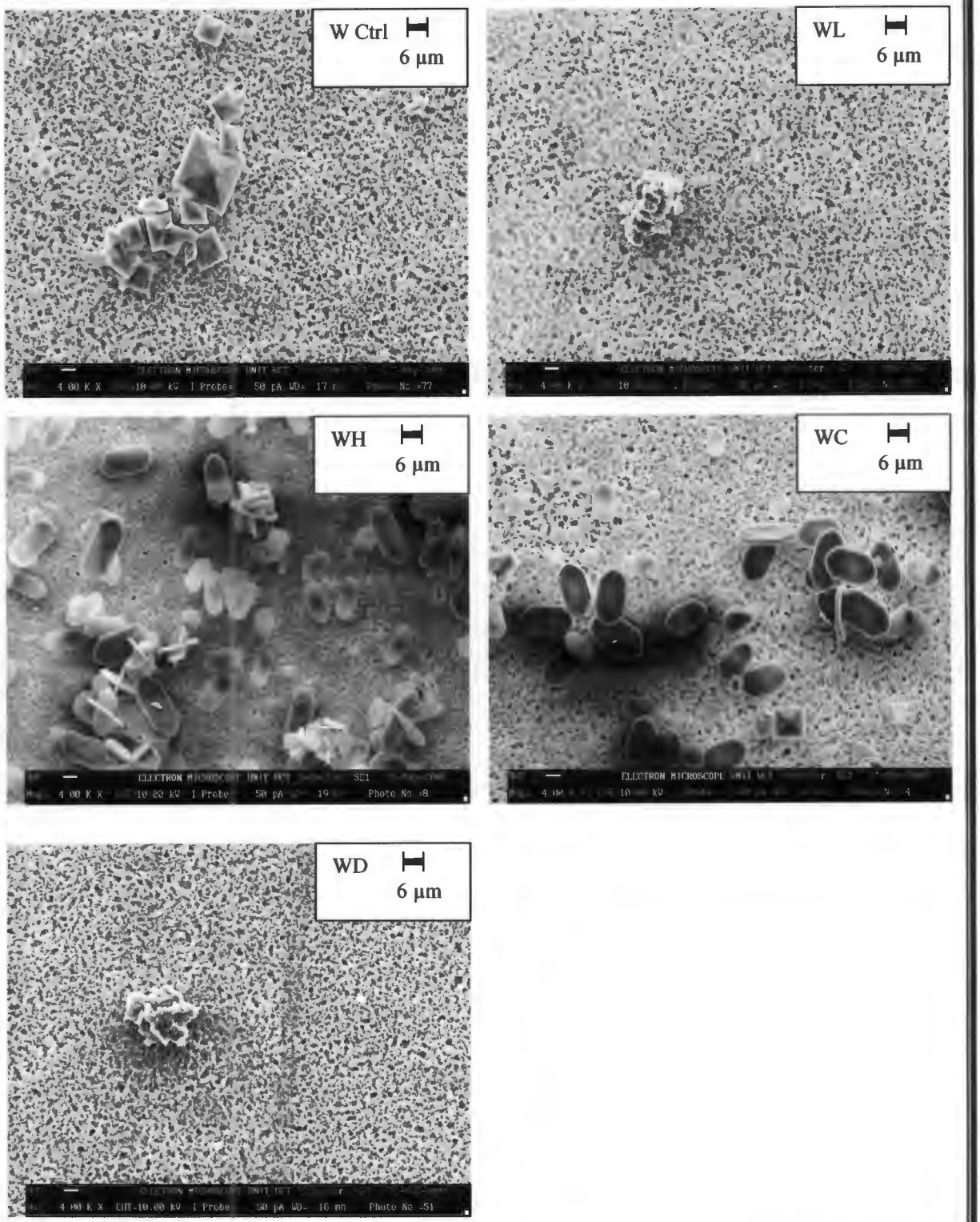


Figure 6.9: Scanning electron micrographs of crystals induced from in samples from white (W) subjects at baseline and at day 7 of each protocol at 4 K magnification. W Ctrl (Baseline); WL (Low NaCl); WH (High NaCl); WC (Sodium Bicarbonate) and WD (Sodium citrate).

Comment

It is noted that the various protocols culminated in a change of morphology from COD to COM in white subjects while in black subjects the morphology was consistently that of the monohydrate. Two other differences were observed in the urines of the two ethnic groups. The first occurred after the L protocol in which several crystals occurred in urine from black subjects but virtually none in that from white subjects. The second difference occurred after the C protocol which culminated in fewer crystals in the urine of black subjects.

Although the reasons for these differences are not immediately apparent, their mere existence is important in the context of the general hypothesis that the two ethnic groups respond differently to the various salt challenges.

[^{14}C]-Oxalate crystal deposition kinetics

The mean percentage plots of ^{14}C-oxalate precipitated as a function of time by urine samples from black (n = 12) and white (n = 12) subjects at baseline and on days 5 and 7 of each salt protocol are shown in Figures 6.10 (a) and (b), respectively. Individual plots are in Appendix 4, Figures 4.3 and 4.4. The mean values and standard errors from which the graph was plotted and the standard errors (SE) are shown in Table 6.22. Comments on the results are offered on page 174.

Table 6.22: Mean values for % ^{14}C-oxalate precipitated after 120 minutes, standard error and p-values in urine samples from black (n = 12) and white (n = 12) subjects at baseline and at Day 5 and Day 7 of each protocol.

Sample	B ± SE	B: Baseline vs. Protocol p-values	W ± SE	W: Baseline vs. Protocol p-values	B vs. W p-values
Baseline	24 ± 2.34		29 ± 2.34		
L5	30 ± 3.21		39 ± 3.21		
L7	18 ± 2.15		28 ± 2.15		
H5	35 ± 3.38		45 ± 3.38	**< 0.05***	
H7	24 ± 2.39		32 ± 2.39		
C5	22 ± 1.58		17 ± 1.58		
*C7	10 ± 0.99	**< 0.05***	21 ± 0.99		**< 0.05***
D5	20 ± 1.56		17 ± 1.56	**< 0.05***	
*D7	11 ± 1.87	**< 0.05***	22 ± 1.87		**< 0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

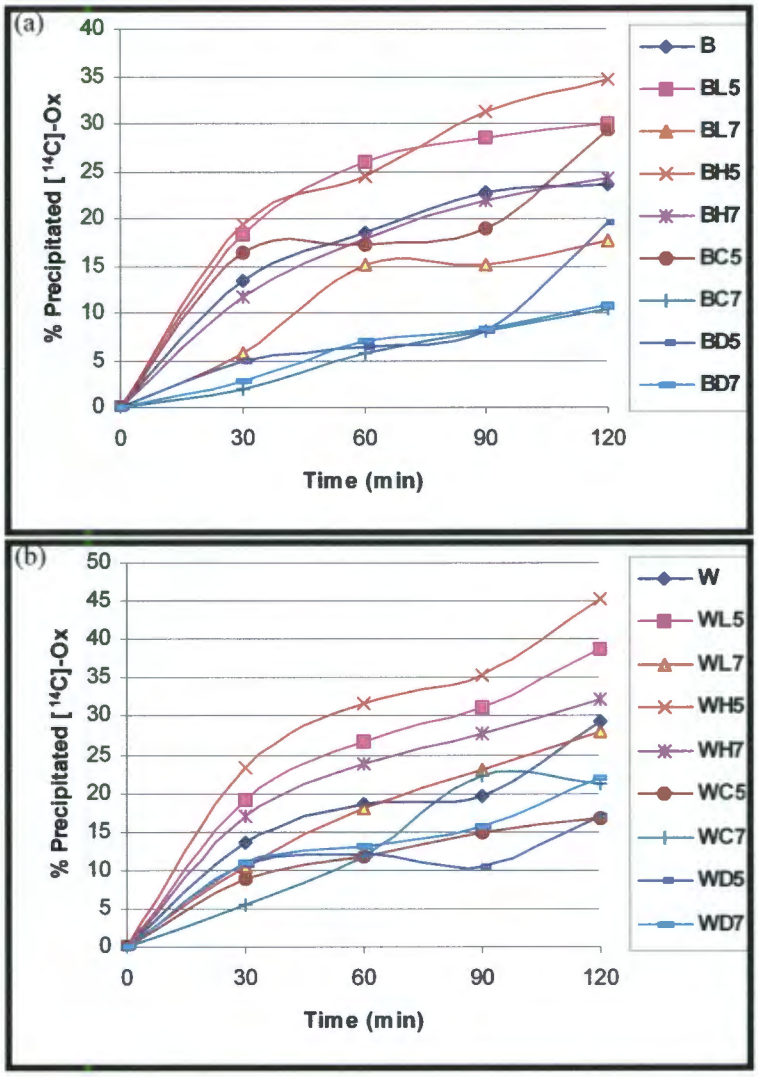


Figure 6.10: Mean [^{14}C]-Oxalate deposited by urine samples from (a) black (b) and white subjects at baseline and day 5 and day 7 of each protocol.

Comment

The sodium chloride protocols tended to increase the deposition kinetics while the sodium bicarbonate and sodium citrate protocols tended to decrease the kinetics in both groups, relative to their respective baseline values.

Qualitative consideration of these results within the two groups shows several interesting features. Firstly, the percentage of ^{14}C-oxalate deposited at baseline is lower in blacks than in white subjects. This relationship is maintained for most of the protocols except C5 and D5. Secondly, the same increase or decrease (relative to their respective baseline values) occurred in the black and white groups for all protocols (except H7 where there was only a small change in whites and no change in blacks).

Of greater interest however, are the quantitative comparisons which show highly significant decreases in black subjects for C7 (from 24 % to 10 %) and D7 (from 24 % to 11 %) while less dramatic differences are observed in white subjects (from 29 % to 21 % and to 22 % respectively).

The qualitative increase in the percentage deposition of crystals containing ^{14}C oxalate in both groups after administration of the low and high NaCl protocols suggests that subtle changes in the urine composition, undetected by urine analyses, must have occurred. A similar argument is valid for the observed changes after the other protocols. These small compositional changes are likely to have been in the urinary calcium. In the case of the NaCl diets, it can be speculated that this parameter increased (due to the physiological action of sodium) while in the case of the sodium bicarbonate and sodium citrate protocols, it can be speculated that this parameter decreased due to pH and complexation effects.

The quantitative results again demonstrate that blacks and whites subjects respond differently to salt challenges. While the groups have had a favourable response (which is expected after administration of sodium bicarbonate or sodium citrate), it was significantly greater in black subjects.

CaOx Crystal Aggregation

The mean percentage inhibition of CaOx crystal aggregation in urine samples from black (n = 20) and white (n = 19) subjects at baseline and at day 5 and day 7 for each protocol are shown in Table 6.23 and the corresponding histogram is presented in Figure 6.11. Individual data are shown in Appendix 4, Tables 4.7 and 4.8.

Qualitatively, the urine samples from black subjects displayed relatively higher percentages of inhibition of CaOx crystal aggregation than those from white subjects after each protocol except for H5. It is noted that in black subjects, one of the protocols resulted in increased inhibition of aggregation relative to baseline (D5) while no such effect occurred with any of the protocols in white subjects.

Table 6.23: Mean percentage of inhibition of CaOx crystal aggregation, standard errors and p-values in urine from black (n = 20) and white (n = 19) subjects at baseline and at day 5 and day 7 of each protocol.

Sample	B ± SE	B: Baseline vs. Protocol (p > 0.05)	W ± SE	W: Baseline vs. Protocol (p > 0.05)	B vs. W p-value
Protocol	76 ± 5.01		73 ± 5.01		
L5	73 ± 3.52		69 ± 3.52		
L7	76 ± 4.19		70 ± 4.19		
H5	68 ± 4.37		73 ± 4.37		
H7	75 ± 3.45		70 ± 3.45		
C5	75 ± 3.29		71 ± 3.29		
C7	73 ± 3.29		70 ± 3.29		
*D5	81 ± 3.33		72 ± 3.33		**< 0.05***
D7	75 ± 3.29		72 ± 3.29		

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

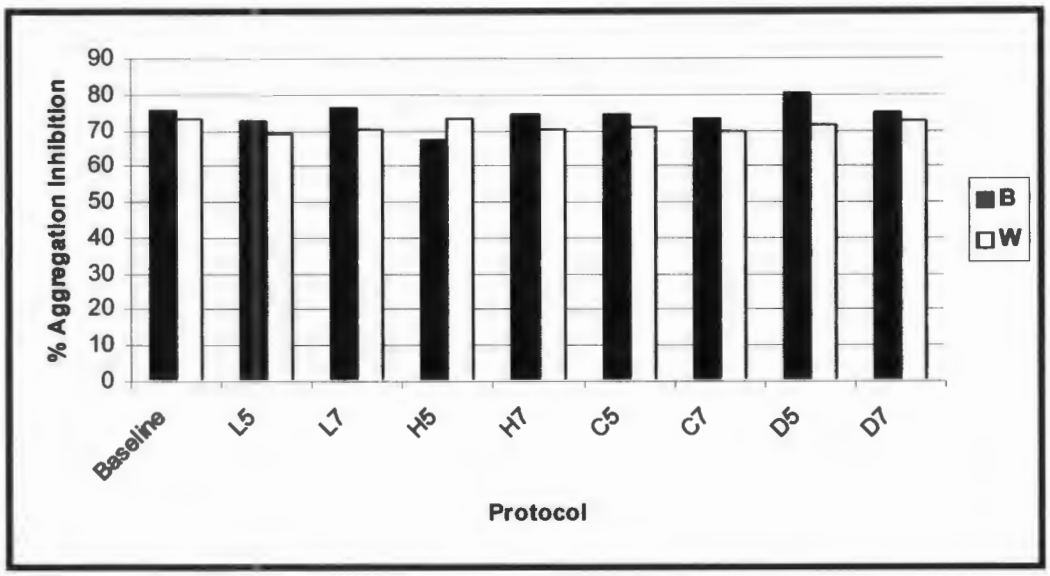


Figure 6.11: Mean percentage inhibition of aggregation at baseline and at Day 5 and Day 7 of each protocol in both black (B) and white (W) subjects.

B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.

Comment

The qualitative results show that inhibition of CaOx crystal aggregation occurs to a greater extent in the urine of black subjects than in white subjects. This is consistent with particle size, SEM and ^{14}C-oxalate deposition results. The solitary quantitative difference between the groups again demonstrates that the two groups handle salt challenges differently and urines from black subjects have a superior ability to inhibit against CaOx crystal aggregation.

CaOx Crystal Nucleation

The mean percentages of inhibition of CaOx crystal nucleation induced by urine samples from black (n = 20) and white (n = 19) subjects at baseline and at day 5 and day 7 of each protocol are shown in Table 6.24 and the corresponding histogram is presented in Figure 6.12. Individual data are shown in Appendix 4, Tables 4.9 and 4.10.

Qualitatively, the samples from black subjects showed a relatively higher inhibition of nucleation for all protocols. Quantitatively, protocols C7 and D7 produced significantly different effects in black and white subjects.

Table 6.24: Mean percentages of nucleation inhibition, standard errors and p-values by urine from black (n = 20) and white (n = 19) subjects at baseline and at day 5 and day 7 of each salt.

Sample	B ± SE	B: Baseline vs. Protocol (p > 0.05)	W ± SE	W: Baseline vs. Protocol (p > 0.05)	B vs. W p-values
Protocol	74 ± 4.88		65 ± 4.88		
L5	78 ± 4.08		72 ± 4.08		
L7	69 ± 3.61		69 ± 3.61		
H5	79 ± 4.25		73 ± 4.25		
H7	72 ± 3.54		66 ± 3.54		
C5	77 ± 3.38		68 ± 3.38		
*C7	79 ± 3.38		59 ± 3.38		**< 0.05***
D5	79 ± 3.42		70 ± 3.42		
*D7	75 ± 3.39		60 ± 3.39		**< 0.05***

** Difference is statistically significant: p ≤ 0.05, B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.*

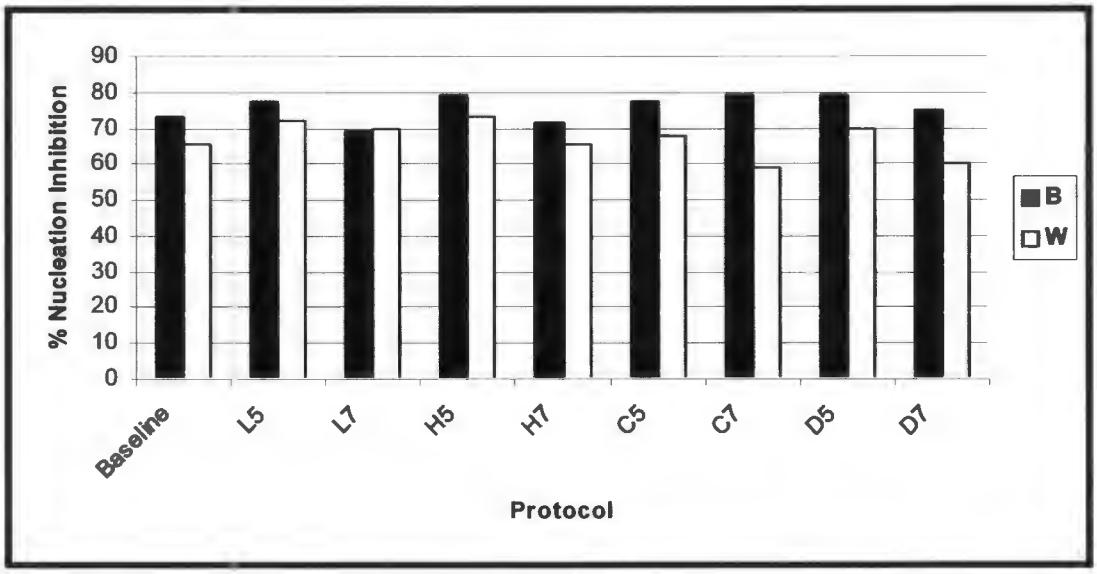


Figure 6.12: Mean percentage inhibition of nucleation before and at Day 5 and Day 7 of each protocol in both black (B) and white (W) subjects.

B (Urine sample from black subjects); W (Urine sample from white subjects). L5 and L7: day 5 and day 7 of low NaCl; H5 and H7: day 5 and day 7 of high NaCl; C5 and C7: day 5 and day 7 of $NaHCO_3$; D5 and D7: day 5 and day 7 of citrate.

Comment

Inhibition of CaOx crystal nucleation occurs to a superior extent in the urine of black subjects than that of white subjects. Despite the non-significant changes, sodium bicarbonate and sodium citrate inhibited CaOx crystal nucleation in both groups.

6.4.3 Urinary Deoxypyridinoline (DPD) and serum Osteocalcin (OC) Analyses

Urinary Deoxypyridinoline (DPD) Concentration

Table 6.25 shows the concentration of urine DPD in samples from black (n = 8) and white (n = 8) subject's baseline and at day 8 of each protocol. Individual values are in Appendix 4, Tables 4.11 and 4.12. There were no significant changes in DPD levels for all the salts in both race groups, and therefore no p-values were tabulated.

Table 6.25: Mean urinary DPD levels (nmol/L), standard errors and percentage changes in samples from black (n = 8) and white (n = 8) subjects at baseline and at day 8 of each protocol.

Protocol	B: Conc. Urinary DPD (nmol/L)	(% decrease/increase wrt baseline)	W: Conc. Urinary DPD (nmol/L)	(% decrease/increase wrt baseline)	B vs. W (% difference)
Baseline	4.63 ± 1.01		4.84 ± 1.01		4 %
Low NaCl (L)	5.41 ± 0.98	14 %	5.79 ± 0.98	16 %	6 %
High NaCl (H)	5.69 ± 1.12	18 %	6.73 ± 1.12	28 %	16 %
$NaHCO_3$ (C)	5.57 ± 0.70	17 %	6.06 ± 0.70	20 %	8 %
Na-Citrate (D)	4.00 ± 0.71	-14 %	5.72 ± 0.71	16 %	28 %

Intergroup comparisons show that that urinary DPD levels are lower in blacks than in whites at baseline and after each salt protocol. Within group comparisons show that urinary DPD levels increased in whites after all four salt protocols, while in blacks, increases occurred after low NaCl (L), high NaCl (H), and sodium bicarbonate (C) protocols but decreased after the sodium citrate (D) protocol. Moreover, the percentage increases in DPD levels after low salt, high NaCl and sodium bicarbonate protocols were generally higher in whites than in blacks, particularly after the high NaCl protocol.

Comment

The lower DPD levels in blacks are consistent with lower bone resorption and lower urinary calcium in this ethnic group. The different qualitative and quantitative effects which occurred between the groups after the various protocols again draws attention to the possibility of different handling mechanisms within the two race groups. Within group comparisons show that all the protocols, except D raised urinary DPD, suggesting that they promote bone resorption irrespective of the race group.

Serum Osteocalcin (OC) concentration

Table 6.26 shows the concentration of serum OC in samples from black (n = 8) and white (n = 8) subjects at baseline and at day 8 of each protocol. Individual values are presented in Appendix 4, Tables 4.13 and 4.14. The OC level is 24 % lower in blacks than whites at baseline. After low NaCl, OC is 8 % higher and 8 % lower after sodium citrate ingestion in whites than blacks. OC is favourably increased in blacks after each salt and even relatively higher after sodium citrate compared to the other three protocols. All the protocols except for low NaCl resulted in an unfavourable decrease in OC levels in whites. There were no significant changes in OC levels for all the protocols in both race groups, and therefore no p-values are tabulated.

Table 6.26: Mean serum OC levels (ng/mL), standard errors and percentage changes in serum from black and white subjects at baseline and at day 8 of each protocol.

Protocol	B: Conc. Serum OC (ng/mL)	(% decrease/increase wrt baseline)	W: Conc. Serum OC (ng/mL)	(% decrease/increase wrt baseline)	B vs. W (% difference)
Baseline	9.17 ± 1.07		12.07 ± 1.07		24 %
Low NaCl (L)	11.84 ± 1.67	22 %	12.90 ± 1.67	6 %	8 %
High NaCl (H)	11.57 ± 2.05	21 %	11.47 ± 2.05	-5 %	1 %
$NaHCO_3$ (C)	12.66 ± 2.52	28 %	10.40 ± 2.52	-13 %	18 %
Na-Citrate (D)	12.89 ± 1.99	41 %	11.80 ± 1.99	-2 %	8 %

Comment

The lower OC levels in blacks (24 %) compared to their white counterparts at baseline are consistent with the lower bone formation reported by Delmas et al. (2000) in the former race group. An increase in OC levels in the black group after all the protocols demonstrates that these protocols (including NaCl) help in regulation and absorption of calcium whereas they tend not to be as effective in whites. This again supports the lower urinary excretion in urine samples from black subjects reported in this study. This observation demonstrates that different handling mechanisms of lithogenic and anti-lithogenic challenges exist in the two South African race groups.

6.4 DISCUSSION

As mentioned in the introductory section of this chapter, the strong positive correlation between urinary calcium and sodium intake is well documented (McCarron et al. 1981; Bresleau et al. 1982; Silver et al. 1983; Burtis et al. 1994; Massey and Whiting 1996; Lin et al. 2003; Massey 2005). However, this effect was not observed in the present study, possibly because the duration of the trial was too short (7 days). Nevertheless, it is of importance to note that high NaCl intake in this study induced an unexpected and counter-intuitive significant decrease in urinary free calcium in samples from black subjects. In addition, the occurrence of some effects in one race group but not the other demonstrates different handling mechanisms of an ingested NaCl challenge in the two South African race groups. These findings are in agreement with the reported studies in other countries (such as Indiana, USA and Europe) comparing racial differences in calcium retention, that race is a major determinant of calcium retention (Kleerekoper et al. 1994; Bryant et al. 2003; Wigertz et al. 2005). With increasing dietary salt, calcium retention is reported to be greater in blacks than in whites; this is attributed to greater calcium absorption and less urinary excretion of calcium in the former group (Kleerekoper et al. 1994; Bryant et al. 2003; Wigertz et al. 2005; Hui et al. 2003).

Since other sodium salts (such as bicarbonate and citrate) induced different urinary effects relative to sodium chloride in this and other studies (Sakhaee et al. 1983; Lemann et al. 1989; Morris and Sebastian 2002), it has been suggested that the anion bound to sodium is as important as sodium itself. Therefore, the chloride anion bound to sodium is as important as sodium in inducing hypercalciuria while the bicarbonate (Lemann et al. 1979; 1986; 1989; Morris and Sebastian 2002) and citrate (Sakhaee et al. 1983) ions help in reducing this condition.

Supplemental bicarbonate and citrate induced a favourable significant decrease in urinary total calcium, free calcium, BRI, RS of CaOx and the RS of uric acid in both groups. Decreases in urinary calcium excretion following sodium bicarbonate and citrate supplementation have been reported by several authors (Lemann et al. 1965; Lutz 1984; Sakhaee et al. 1983). It is suggested that these two anions (bicarbonate and citrate) promote calcium retention, thereby negating the calcium lost by sodium. This finding has been demonstrated in Kurtz et al.'s study where equimolar amounts of NaCl and sodium citrate

were administered (Kurtz et al. 1983). The NaCl study resulted in increased urinary calcium excretion whereas sodium citrate had no effect on this parameter (Kurtz et al. 1983). Some researchers have demonstrated that urinary calcium excretion decreases following administration of sodium bicarbonate (Lemann et al. 1965; Lutz 1984).

Another important effect of sodium bicarbonate and citrate administration in this present study is a significant increase in pH. Pak et al. (1994) attribute this increase in pH to the *in vivo* oxidation of citrate to bicarbonate which results in disturbances in the acid-base balance of the urine. An increase in pH following administration of sodium bicarbonate and sodium citrate is considered as the main factor in the success of these protocols in the management of CaOx stone formation (Pak et al. 1985; Pak and Fuller 1986; Lemann et al. 1989; Borghi et al. 2002; Morris and Sebastian 2002). Increased urinary pH is associated with an increase in inhibition of CaOx crystallization (Pak 1994). This is due to the fact that at a higher pH, more phosphate and citrate ions are dissociated thus promoting the complexation of calcium and citrate ions which in turn lowers the relative supersaturation of CaOx (Pak 1994). As mentioned previously, Rodgers et al. (2005) have recently demonstrated the theoretical existence of a new calcium-citrate-phosphate complex which significantly lowers the RS of CaOx. In support of the notion that a higher pH is favourable, it has been previously reported that CaOx stone formers have significantly lower urinary pH values compared to healthy controls (Pak et al. 2002; Tiselius 2003; Hess 2006).

To support the physicochemical data, crystallization experiments were conducted to determine whether the urines from the two groups behaved differently after the various protocols even though compositional differences were not necessarily detected.

The Coulter Counter data showed a decrease in particle number and size in urines from black subjects at baseline and after all the protocols, thereby demonstrating a lower risk of CaOx crystal nucleation, aggregation and growth in this group whereas promotion of these unfavourable mechanisms was noted in samples from white subjects.

These data were supported by scanning electron microscopy which also provided some interesting results. COD crystals precipitated at baseline were in agreement with the higher urinary calcium concentration reported in the samples from whites in this study. It has been previously reported that whites have very higher urinary calcium concentrations

(Goulding 1990; Nordin et al. 1992; Burtis et al. 1994), and these high concentrations favour the formation of COD crystals (Webber et al. 2002). The change in morphology from COM to COD crystals in samples from white subjects following sodium citrate ingestion is considered beneficial since the latter are less adherent to renal tubular cells (Wesson et al. 1998). A decrease in crystal size in samples from black subjects after sodium bicarbonate and sodium citrate administration is in agreement with the previously reported results that these protocols reduce and retard CaOx crystal growth (Pak et al. 1983; Lemann et al. 1989; Laube et al. 2002 (a); Morris et al. 2002). The lower ^{14}C-oxalate deposition in blacks compared to whites at baseline and after all of the protocols is in agreement with particle size and SEM data. This could offer an explanation to the rarity of kidney stone disease in the black group. This also indicates that black and white subjects respond differently to dietary lithogenic and anti-lithogenic challenges.

Spectrophotometric analyses also demonstrated a greater extent of inhibition of CaOx crystal nucleation and aggregation in samples from black subjects than those from white subjects. Despite the non-significant differences, the slight increase in the extent of inhibition of nucleation in blacks after all the protocols except for high NaCl indicates the potency of these protocols in reducing the risk of stone formation. These observation are in agreement with those reported by several authors that supplemental bicarbonate and citrate retard CaOx crystal nucleation, aggregation and growth (Pak et al. 1983; Lemann et al. 1989; Kok et al. 1986; Pak 1994; Laube et al. 2002 (a); Morris et al. 2002; Allie-Hamdulay and Rodgers 2005).

In retrospect, the choice of Citro-Soda as a source of citrate may not have been entirely appropriate as it contains bicarbonate which might have confounded the influence of citrate itself. Nevertheless, the additive effect, if any, is of interest.

As explained in Chapter 1, osteoporosis just like kidney stone disease is a multi-factorial disease. There is a profound link between these two diseases as they both depend on calcium absorption and excretion. High urinary calcium excretion subsequently reduces calcium transportation into bone which has a negative impact on skeletal development which adversely leads to osteoporosis. Furthermore, elevated urinary calcium results in a high risk of it complexing with oxalate to form an insoluble complex of CaOx which is the main component of kidney stones. Maximal positive balance between calcium intake and obligatory losses of calcium are required for the attainment of peak bone mass and for the

reduction of kidney stone formation risk. Indeed, patients suffering from osteoporosis due to renal tubular acidosis have been reported as having kidney stones (Weger et al. 1999; Roudsari et al. 2005).

As one of the objectives of this study, the effect of the already discussed three sodium salts on bone formation and bone resorption in both black and white South African male subjects was investigated. However, baseline values of these markers are of interest. The lower DPD and OC levels in blacks demonstrate fundamental physiological differences between the groups which ultimately determine urinary calcium and stone formation.

Several studies have investigated the effect of sodium chloride on bone turnover markers but conflicting results have been reported (Goulding 1981; Goulding and Lim, 1983; Shortt and Flynn, 1990; Matkovic, et al. 1995; Evans et al. 1997; Lietz et al. 1997; Ginty et al. 1998; Sellmeyer et al. 2002; Lin et al. 2003; Massey 2005; Wigertz et al. 2005). Several authors have reported a decrease in OC levels (bone formation marker) following a high sodium intake and no change in bone resorption markers (Evans et al. 1997; Sellmeyer et al. 2002; Lin et al. 2003). Others have reported an increase in OC levels and a decrease in bone resorption markers (Sebastian et al. 1994; Wigertz et al. 2005). Not unexpectedly, some investigators have shown no effect on bone formation and resorption markers (Lietz et al. 1997; Ginty et al. 1998).

The qualitatively smaller increases in urinary DPD levels in blacks after low NaCl, high NaCl and sodium bicarbonate protocols are indicative of a weaker bone resorption response to these challenges than in whites. On the other hand increases in serum OC in blacks after high NaCl, sodium bicarbonate and sodium citrate protocols but corresponding decreases in whites surprisingly demonstrate a bone formation response in the former. Thus, the notion of different renal handling mechanisms in the two race groups is again apparent. This supports the findings of several researchers who believe that race is a major determinant of calcium retention (Kleerekoper et al. 1994; Bryant et al. 2003; Hui et al. 2003; Wigertz et al. 2005). Indeed, blacks are reported to have significantly higher bone mineral density and bone mineral content than whites which could be due to higher calcium retention by the former group (Wigertz et al. 2005).

CHAPTER SEVEN

CONCLUDING COMMENTS

On the basis of the well established observation that the South African black population rarely forms kidney stones (Modlin 1967; Whalley et al. 1998; Pinnock et al. 2004), the present thesis was undertaken to investigate various aspects of this phenomenon, with a view to identifying the factors which afford this protection.

The present study corroborated previous findings (Lewandowski et al. 2001; Rodgers and Lewandowski 2002) that the urinary composition and CaOx MSL data alone do not explain rarity of kidney stone disease in the black group. Therefore crystallization experiments measuring as particle formation kinetics, ^{14}C-oxalate deposition, CaOx crystal aggregation and nucleation were conducted. These studies were also supplemented with SEM to view the extent of crystallization and the morphology of the crystals precipitated. The zeta potential of crystals was also measured.

Crystallization studies conducted in prefiltered, ultrafiltered and concentrated urine portions from black and white subjects demonstrated that macromolecules in the urine of the former were more effective at inhibiting CaOx crystal deposition and aggregation. This result is in agreement with that demonstrated by Tiselius et al. (1995) and Asplin (1999) that healthy men show greater CaOx aggregation inhibition than stone prone men. This demonstrated the inhibitory role of macromolecules and gave some explanation as to the stone rarity in the black South African population.

Investigation of the crystal matrix extract included in COD derived from the urines of both race groups showed that the extract from black subjects (BCME) was a superior inhibitor of CaOx crystallization processes. The total protein content in BCME was higher than in WCME. SDS-PAGE and Western Blotting revealed OPN to be the main intracrystalline protein in both BCME and WCME. This observation is in agreement with the results reported by several authors (Atmani et al. 1996; Webber et al. 2003; Ryall et al. 2005). On the basis of this finding, the inhibitory activity of the CMEs was mainly attributed to OPN.

Complimentary studies on the role of the race groups' respective urine compositions, demonstrated that commercially available OPN performed as an inhibitor to a better extent in the urine from black subjects compared to that from white subjects. This was in support of the observation that OPN is the main intracrystalline protein in COD-CME.

Investigation of the ingestion of three sodium salt protocols gave rise to changes in the urinary biochemical risk factors for CaOx stone formation in both groups. Several of these changes were common to both groups and were generally favourable. In addition to the common changes, several urinary risk factors changed in the one group but not in the other. The significantly lower urinary calcium in baseline samples from black subjects compared to those from their white counterparts was in agreement with the previously reported data in South Africa (Modlin 1967; Whalley et al. 1998; Rodgers and Lewandowski 2002). Of importance however, was an indication that the changes in black subjects were more prominent in providing physicochemical protection against kidney stones. An unexpected and counter-intuitive significant decrease in free calcium in urine samples from black subjects, but not in those from white subjects after NaCl ingestion was also noteworthy. These findings are in agreement with other studies that reported racial differences in calcium retention, with retention being greater in blacks than in whites (Kleerekoper et al. 1994; Bryant et al. 2003; Wigertz et al. 2005). This is attributed to greater calcium absorption and less urinary excretion of calcium in the black group (Kleerekoper et al. 1994; Bryant et al. 2003; Wigertz et al. 2005; Hui et al. 2003). This provides important evidence in support of the notion that different physiological handling mechanisms of lithogenic and antilithogenic dietary challenges occur in the two race groups. Similarly, the observation of qualitative and quantitative differences in bone turnover markers in both groups following the ingestion of different sodium salts, provides further support of this notion.

Thus, the overall results of the various studies presented in this thesis have demonstrated several superior physicochemical, biochemical and physiological protective mechanisms against CaOx kidney stones in black South African subjects compared to their white compatriots. These mechanisms are likely to be important contributory factors towards accounting for the rarity of urolithiasis in this race group.

CHAPTER EIGHT

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