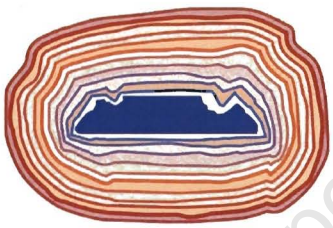
Investigation of the Crystallisation Inhibitory Properties of Albumin Isolated from the Urine of Black and White South Africans



by

Priscilla D.N.A Mensah

Supervisors: Professor A. Rodgers & Dr E. Sturrock

I dedicate this thesis to my parents Samuel and Lucy, my brothers Samuel Jnr. and Abraham and my sister Sarah who are my pillars of support. Thank you for always encouraging me to give my best.

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ABSTRACT

INTRODUCTION: Stone incidence in South African blacks is extremely rare. The hypothesis to be tested is whether this apparent immunity can be attributed to the presence of more potent inhibitors of calcium oxalate (CaOx) crystallisation in urine from blacks compared to those occurring in the urine of white subjects. Urinary proteins have been shown to be inhibitors. This study investigated the crystallisation inhibitory properties of albumin isolated from the urine of healthy black (BA) and healthy white (WA) South Africans as well as those of human serum albumin (HSA). Albumin was selected because previous studies on other population groups had revealed that the protein inhibits CaOx aggregation. This observation is significant because crystal aggregation is considered to be more important for stone formation than crystal nucleation or growth. **METHODS:** Albumin was isolated from the urine of healthy black and healthy white subjects by immunoaffinity chromatography. Commercial HSA was obtained from Sigma. The purity of each protein was checked using sodium dodecyl sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting. Crystallisation experiments were performed in ultrafiltered urine from the two race groups. These experiments included determination of CaOx metastable limit, measuring the kinetics of particle formation in supersaturated ultrafiltered urine, Coulter Counter measurements of particle volume-size distributions and crystal deposition kinetics using labelled [^{14}C]-oxalate. X-ray diffraction analysis was used to confirm the purity of calcium oxalate monohydrate (COM) crystals prepared *in vitro* and to confirm the composition of calcium oxalate crystals induced in urine samples as part of the protocol to isolate albumin. Aggregation inhibition of COM crystal slurries by BA, WA and HSA was studied *in vitro* using zeta potentials and sedimentation rates. **RESULTS:** The concentration on urinary albumin was determined to be 0.802 mg/*l* in blacks and 0.743 mg/*l* in whites. The metastable limit of ultrafiltered urine from blacks (BUF) and whites (WUF) was 0.100 mol/dm^3 and 0.045 mol/dm^3 respectively. Data acquired by spectrophotometry, Coulter Counter and [^{14}C]-oxalate deposition were considered in concert and demonstrated that albumin from both race groups inhibits the crystallisation of CaOx. The data also suggested the occurrence of independent protein co-precipitation i.e. adsorption of the protein directly onto CaOx crystal surfaces. X-ray diffraction data demonstrated that urine from blacks and whites contained calcium oxalate dihydrate (COD) crystals and a mixture of COM and COD respectively. Zeta potential measurements and sedimentation rates were used to

investigate the effect of albumin on calcium oxalate monohydrate (COM) crystal aggregation. The zeta potential of COM crystal slurries increased in the presence of all three proteins. Values followed the trend BA (-19.3 mV), WA (-16.9 mV), HSA (-14.2 mV) and COM control crystals (-13.0 mV). The percentage inhibition of aggregation by HSA and WA, as determined from sedimentation experiments, was not significantly different, (31.3 % versus 35.1 % respectively) while inhibition of COM crystal aggregation by BA was significantly higher at 50 %. **CONCLUSION:** The results have shown that BUF can tolerate higher levels of supersaturation without the formation of CaOx crystals, compared to WUF. Results obtained by spectrophotometry, Coulter Counter and [^{14}C]-oxalate deposition demonstrated that albumin, regardless of its source, inhibits the rate of formation of CaOx and that BA is more efficacious in this regard. It is suggested that the formation of COD over COM in the urine of healthy black subjects would be advantageous. Measurements of zeta potentials and sedimentation rates confirmed that albumin, irrespective of its origin, is an inhibitor of COM crystal aggregation and that BA is superior to WA and HSA in this regard. It was concluded that albumin may play a contributory role to the overall immunity of the black population group in South Africa to urolithiasis.

CHAPTER 1: INTRODUCTION

1.1 Kidney Stone Disease

The human body is a network of sophisticated systems working harmoniously together to sustain life. We are often oblivious to how well-coordinated these systems are until something goes wrong. Many of the chemical processes in the body release toxins into the bloodstream. If allowed to remain, these can lead to serious complications, even death. They have to be continuously filtered and removed. This filtering is one of the principal functions of the kidneys. The nephron is the basic filtration unit. There are over a million nephrons in each kidney (Davidson, 1981).

Briefly, as blood is forced through the glomerulus, water, waste products, salts and other molecules are filtered into Bowman's capsule. The resulting glomerular filtrate now flows down in the proximal convoluted tubules, where re-absorption of water, glucose, other nutrients, sodium and other ions occurs. Re-absorption continues in the loop of Henle, distal convoluted tubules and collecting tubules. As the fluid moves through the distal and collecting ducts, cells in the tubule wall release additional secretions into it including ammonia, potassium, hydrogen ions, urea, uric acid and excess water (Davidson, 1981). The final product is urine.

It is in this biochemical milieu that renal calculi develop. Urinary stones are polycrystalline masses found anywhere along the urinary tract – kidneys, ureters, bladder and urethra as illustrated in Fig 1 (Gill et al., 1974; Hess et al., 1996). The term nephrolithiasis is used to refer to the whole clinical picture due to the formation and passage in the urinary tract of these calculi or stones (Ramello et al., 2000).

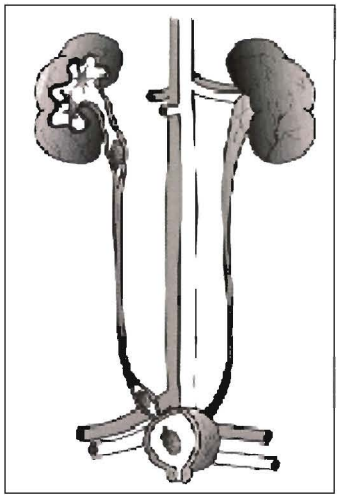


Fig 1: *Urinary stones form anywhere along the urinary tract taken from www.transweb.org*

The different types of kidney stones are composed of calcium oxalate, calcium phosphate, uric acid, cystine or magnesium ammonium phosphate (Coe et al., 1980). Calcium oxalate is the major constituent of over 80 % of urinary calculi, and whewellite (calcium oxalate monohydrate) forms the central core (probably the nidus) in 70 % of upper urinary tract calculi (Elliot, 1973; Gill et al., 1974). Some authors (Grases et al., 1988) argue that a closer examination of such a nidus will reveal calcium phosphate at the center. Crystallisation of calcium oxalate and calcium phosphate from urine, therefore, would seem to be obligatory for stone disease. An essential condition for a crystal to grow from a solution is supersaturation (Hess et al., 1996; Kavanagh, 2000).

There are six accepted factors that have been shown to increase the risk of calcium stones. These risk factors are considered to affect the degree of saturation of urine with calcium oxalate and calcium phosphate. They are, a low urine volume; increases in the excretion of calcium, oxalate and uric acid; decreases in the excretion of the glycosaminoglycan (GAG) inhibitors of crystallisation and fluctuations in pH (Robertson et al., 1981). There is debate about whether it is the increase or decrease in pH that increases the risk of stone formation. In the model of calcium stone formation, illustrated in Fig 2, the authors proposed that an increase in pH favours stone formation because solubility of CaOx decreases with increasing pH (Robertson et al., 1981; Robertson et al., 1983). However, subsequent studies have demonstrated that the inhibition of calcium oxalate crystal growth in urine from stone-formers increased with increasing pH, implying that consumption of foods or beverages that decrease pH would represent an increased risk of stone formation (Tiselius, 1981; Hess et al., 1999; Rodgers, 1999). At a higher pH, more phosphate and citrate ions

become dissociated; this augments the complexation of calcium thereby lowering the urinary saturation of stone forming salts (Pak, 1994).

The risk factor model for calcium oxalate stone formation proposed by Robertson and colleagues (1981), shows how epidemiological factors can increase or decrease the effect of the above mentioned risk factors (Fig 2). These risk factors in turn affect urinary supersaturation with respect to calcium oxalate (CaOx) or calcium phosphate (CaP). If the overall effect is an increase in supersaturation and a decrease in the activity of urinary inhibitors, then the result will be rapid crystal formation and growth which will lead to stone formation (Robertson et al., 1981).

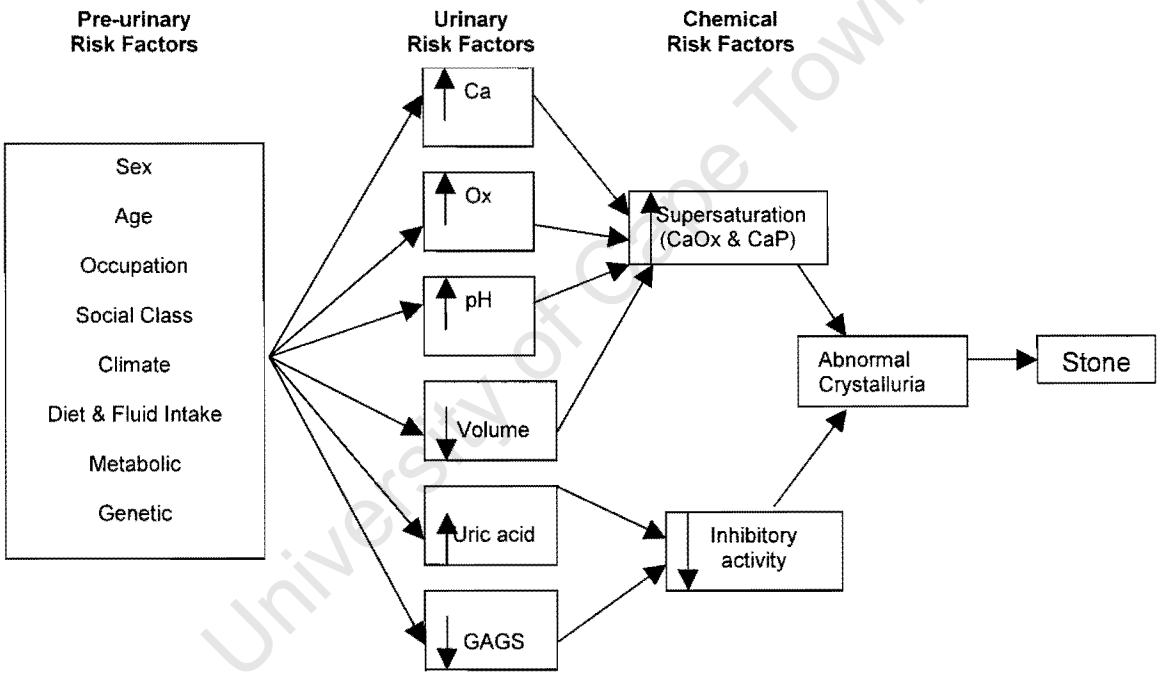


Fig 2: *Model of calcium stone formation (Robertson et al., 1981)*

1.2 Epidemiology of Nephrolithiasis

Kidney stones are one of the most ancient afflictions of mankind. Ancient Babylonians and Egyptians were familiar with stones of the urinary tract. Bladder stones have been discovered in Egyptian mummies (Coe et al., 1980). Today, the varied manifestations of nephrolithiasis provide an interesting epidemiological study from the standpoints of geography, diet, drinking water, profession, age, race and sex.

1.2.1 Geography and Climate

Research into the geographical pathology of stone disease shows that its prevalence is very far from constant (al-Hadramy, 1997; Ramello et al., 2000; Robertson, 2003). The risk of developing nephrolithiasis in normal adults is 1-5 % in Asia, 5-9 % in Europe and in North America it is 12 % in Canada and 13 % in USA (Ramello et al., 2000). Saudi Arabia reportedly has the highest risk at 20.1 % (Robertson et al., 1994). In tropical countries where individuals consume large quantities of local leaves and vegetables which are high in oxalate, the concentration of urinary oxalate is high and, as a result, the ammonium acid urate stones which are commonly formed, contain calcium oxalate as well (Robertson, 2003). Stone composition and location within the urinary tract (bladder or kidney) can differ in different countries and even in the same geographical region, clinical and metabolic patterns of stone disease can change over time. The following examples, extracted from the review by Ramello et al. (2000) illustrate this point. In India, between 1965 and 1985 the incidence of bladder stones dropped from 30 % to 5 %. At the same time, the prevalence of calcium oxalate (CaOx) stones rose from 26 % to 82 %, and that of struvite stones (magnesium ammonium phosphate) diminished from 20 % to 5 %. In Japan, bladder stones decreased from 50 % to 5 % between 1950 and 1985. In Portugal, over a twenty-year period, the frequency of calcium stones rose from 64 % to 82 %, struvite stones decreased from 14 % to 3 % and uric acid stones from 19 % to 12 % (Ramello et al., 2000).

A study in Perth, Western Australia (Bateson, 1973) was carried out to compare the frequency of renal calculi during the hot summer months to its incidence rate during the cold and wet winter. Results showed that the prevalence in summer (46 %) was nearly double that in winter (25 %). This variation was attributed to excessive water loss by perspiration which leads to dehydration in summer. If coupled with inadequate fluid intake, an increase in urine super-saturation due to low urine output increases the risk of crystalluria and kidney stone formation. People who live in hot climates are therefore encouraged to monitor their fluid intake, particularly if they are inclined to form kidney stones (Bateson, 1973).

In a similar study in Leeds (Hallson and Rose, 1977), seasonal crystal incidence was compared with seasonal changes in urinary composition. Crystalluria was taken as an

indicator of urine super-saturation with the material from which the crystals were composed. Individual urine samples from normal subjects and stone-formers with idiopathic hypercalciuria were examined for crystals both qualitatively and quantitatively at 37 °C. The group as a whole showed a rise in incidence of urinary crystals in the summer months. This rise was seen most clearly in overnight urines, collected on rising in the morning, and the patients appeared to be at a risk overnight during the summer. The rise in crystal incidence during the summer was associated with increased creatinine concentrations in the same urine samples and with increased oxalate concentration in 24-hour urine collections (Hallson and Rose, 1977).

1.2.2 Diet

During the past 25 years, several important studies have addressed the role of diet in calcium oxalate nephrolithiasis (Vahlensieck, 1986; Kok et al., 1990; Trinchieri et al., 1991; Hesse et al., 1993; Hess et al., 1994; Goldfarb, 1994; Parivar et al., 1996; Curhan, 1999; Massey et al., 2001; Nguyen et al., 2001; Rodgers et al., 2002; Borghi et al., 2002). An important dietary factor which is linked consistently at all demographic levels with expenditure on food and the occurrence of stones is the intake of animal protein.

The effect of modulating dietary factors on actual urinary supersaturations has been studied in healthy volunteers (Hesse et al., 1993). While leaving the ingested amounts of calcium, oxalate, and fibre unchanged, a reduction in daily intakes of protein (from 85 to 65 g) and purines (from 509 to 219 mg) lowered relative supersaturations of both calcium oxalate and uric acid by 58 % and 85 % respectively (Hesse et al., 1993). A recent study by Hess et al. (1999) went a step further by assessing the direct impact of dietary advice, which primarily consisted of increased fluid intake as well as a reduction in meat protein and salt consumption, on actual urinary composition and supersaturation in male patients with idiopathic calcium urolithiasis (ICSFs). The authors reported that dietary advice which solely focused on individual abnormalities of either urine volume or urea excretion rate (as a marker for meat protein intake) lowers urinary supersaturations by only 19 % in ICSFs. However, in ICSFs in whom dietary advice was able to jointly stimulate increases in urine volume and reductions in meat protein intake, the relative supersaturation of calcium oxalate (RS_{CaOx}) and uric acid (RS_{UA}) decreased (Hess et al., 1999). Thus, consumption of animal protein may distinguish renal stone formers from healthy individuals (Robertson et

al., 1981; Kok et al., 1990; Trinchieri et al., 1991; Giannini et al., 1999; Massey et al., 2001 and Nguyen et al., 2001).

Biochemically, a diet high in animal protein increases the urinary excretion of calcium, oxalate and uric acid as well as lowers citrate excretion, all of which are well known risk factors for idiopathic calcium nephrolithiasis (Hess et al., 1999). It has been reported that in vegetarians, who have a high intake of vegetable protein, compared with animal protein, there is a low incidence of upper urinary tract stones. The reason for this is that a diet low in animal protein reduces the urinary excretion of calcium, oxalate and uric acid, 3 of the 6 main urinary risk factors, so that the overall probability of stone formation is very low (Robertson et al., 1979; Vahlensieck, 1986).

More recently, Massey et al. (2001) studied the effect of substituting equal amounts of dietary protein (beef) for plant protein (legumes, seeds, nuts and grains) on urinary components associated with calcium oxalate precipitability risk. The findings of these authors is in agreement with previous studies and showed that a balanced diet containing moderate amounts of either beef or plant protein coupled with adequate fluid intake is effective in reducing the risk of calcium oxalate stone formation (Vahlensieck, 1986; Hesse et al., 1993; Goldfarb, 1994; Parivar et al., 1996; Giannini et al., 1999; Massey et al., 2001). Therefore, while the risk of stone formation in vegetarians is low, non-vegetarians are afforded protection from stone disease by balancing the amount of ingested animal and plant protein and increasing their fluid intake.

Another important dietary factor which has received a huge amount of attention is that of calcium ingestion. Oral calcium restriction was traditionally recommended for the prevention of calcium nephrolithiasis (Bleich et al., 1979; Robertson, 1987). However, several recent studies have revealed that the restriction of dietary calcium in renal stone formers is not warranted (Curhan et al., 1993; Massey et al., 1993; Messa et al., 1997; Curhan et al., 1997; Heller et al., 1999). Rather, normal dietary calcium intake, along with reduced salt and protein, is now advised (Martini et al., 2000; Massey et al., 2001; Hall, 2002). There is also evidence that the restriction of dietary calcium leads to an enhanced absorption and excretion of oxalate, while an increase in calcium intake results in decreased gastrointestinal absorption of dietary oxalate and lower urinary oxalate concentrations (Curhan et al., 1993; Curhan et al., 1997).

Dietary oxalate restriction has been found to be beneficial in preventing recurrence of calcium oxalate urolithiasis (Pak et al., 1984; Massey, 2003). Foods that are very high in oxalate include spinach, rhubarb, and beets and should be avoided by stone forming patients, as they elevate urinary oxalate excretion. Calcium oxalate stone-formers should also avoid consumption of large quantities of foods containing higher amounts of oxalate such as concentrated wheat bran, chocolate, nuts and berries (Smith, 1991; Massey et al., 1993; Massey et al., 1998). de O G Mendocana et al. (2003) investigated the effect of an oxalate load on urinary oxalate excretion in calcium stone-forming patients. The authors reported that a 2-fold increase in oxalate intake produced a significant 20% increase in oxaluria, confirming previous observations that hyperoxaluric patients will benefit from reduction of dietary oxalate (Tiselius, 1980; Brinkley et al., 1981; Finch et al., 1981; Larsson et al., 1987).

1.2.3 High Fluid Intake

An increase in fluid intake is routinely recommended for patients who have had kidney stones to decrease the likelihood of recurrence (Shuster et al., 1982; Vahlensieck, 1986; Ackermann et al., 1988; Weiss et al., 1992; Coe et al., 1992; Wabner et al., 1993; Curhan et al., 1996; Seltzer et al., 1996; Rodgers, 1997; Curhan et al., 1998; Massey et al., 1998; Rodgers, 1999; Hirvonen et al., 1999; Goldfarb et al., 2000; Honow R et al., 2000; Knight et al., 2003).

Theoretically, a high fluid intake may inhibit stone formation, since it would dilute urine, lower urinary concentration of stone-forming constituents, and reduce urinary saturation of stone salts. However, it was thought that this strategy would also dilute urinary concentration of inhibitors, which normally retard crystallisation of stone salts. It was thus feared that the decline in inhibition might negate the beneficial effect of reduced saturation. These circumstances led Pak et al. (1980) to examine in detail the effect of urinary dilution on the crystallisation of calcium salts.

Urinary dilution was achieved *in vitro* (1 to 2 *l*/d) by addition of water to urine from six patients with renal stones and two normal subjects, and *in vivo* (1.023 to 2.383 *l*/d) by an increased ingestion of distilled water in four patients with nephrolithiasis and three normal

subjects. Both forms of urinary dilution significantly reduced the state of supersaturation of calcium oxalate and calcium phosphate. In addition, the metastable limit of calcium oxalate significantly increased. The limit of metastability is defined as the minimum amount of oxalate necessary to induce nucleation (Ryall et al., 1985). Thus, urinary dilution was found to reduce the tendency for the crystallisation of calcium salts in urine by lowering the level of supersaturation and by increasing the minimum supersaturation needed to elicit spontaneous nucleation of calcium oxalate (Pak et al., 1980).

That an increase in fluid intake can reduce the risk for kidney stones has been confirmed by recent studies (Curhan et al., 1998; Borghi et al., 1999). It is recommended that to avoid recurrence, stone-formers drink a sufficient amount of fluid to attain urine output of 2*l* per day. Increasing fluid intake has been found to prevent recurrence in 60 % of patients with idiopathic calcium urolithiasis (Hosking et al., 1983).

The choice of beverage has also been found to be meaningful since fluid composition directly influences urine composition and crystal formation (Hesse et al., 1993). Although water is most commonly imbibed, recent studies have shown that mineral water containing calcium and magnesium has therapeutic and prophylactic value in calcium oxalate nephrolithiasis (Ackermann et al., 1988; Rodgers 1997). Epidemiological studies by Curhan et al. (1998) suggested that grapefruit juice increased the risk of stone formation. However, recent studies have not confirmed this to be so (Goldfarb et al., 2000; Honow et al., 2000). Similar conflicting studies have been found for alcohol (Vahlensieck, 1986; Hirvonen et al., 1999; Knight et al., 2003). Fluids that have been found to alter risk factors favourably include milk (Coe et al., 1992), orange juice (Coe et al., 1992; Wabner et al., 1993), apple juice (Vahlensieck, 1986; Curhan et al., 1996; Massey et al., 1998), cranberry juice (McHarg et al., 2003), herbal teas (Vahlensieck, 1986; Hesse et al., 1993), and lemonade (Seltzer et al., 1996). Fluids that should be avoided by stone formers include coffee, tea and carbonated cola drinks (Vahlensieck, 1986; Weiss et al., 1992; Rodgers, 1999).

1.2.4 Profession and Stress Levels

Occupational conditions have been found to affect susceptibility to stone disease. Urolithiasis occurs more frequently in pilots than in the ground-service, probably due to

the sedentary nature of their work (Garilevich et al., 1995). Among workers chronically exposed to a hot environment and massive sweating, the prevalence of nephrolithiasis was 8.5 % while only 2.5 % of control subjects developed stone disease (Ramello et al., 2000). Also, beach lifeguards and marathon runners have a higher incidence of urinary calculi due to chronic dehydration and/or low fluid intake (Parivar et al., 1996).

A questionnaire survey was carried out by Pin et al. (1992) to determine the prevalence of urinary stone disease in 406 male workers in several occupations. There were 119 quarry drilling and crusher workers (outdoor, physically active), 77 quarry truck and loader drivers (outdoor, physically inactive), 92 postal deliverymen (outdoor, physically active), 75 postal clerks (indoor, physically inactive) and 43 hospital maintenance workers (indoor, physically active). Urinary stone disease was found to be 5.2 % in outdoor workers compared to 0.85 % in indoor workers. Surprisingly, no increased risk of urolithiasis was apparent in physically inactive workers. Chronic dehydration was considered to be the most important factor for the increased risk of urolithiasis in outdoor workers, and can therefore be easily prevented by increased water intake (Pin et al., 1992). Similar findings were obtained by Borghi et al. (1993) who investigated the prevalence of stone disease and urinary risk factors in machinists chronically exposed to a hot environment and massive sweating. Nephrolithiasis was found in 8.5 % of the entire population of machinists (20 of 236), while the prevalence on the controls working in normal temperature was 2.4 % (4 of 165). This study confirmed that chronic dehydration is a real lithogenic risk factor. Adequate fluid intake is therefore recommended for individuals working in hot environments (Borghi et al., 1993).

A recent study by Najem et al. (1997) suggested a possible explanation for idiopathic hypercalciuria and nephrolithiasis that involves the role of stressful events in simultaneously reducing urinary inhibitors (magnesium, citrate), promoting hyperoxaluria and lowering urinary volume. The stressors include those life events that the subjects perceived as highly stressful and inflicted upon them an intense emotional impact with apprehension and distress for at least one week in duration. Studies of 24-hour urine specimens showed that lithogenic urinary constituents (calcium, oxalate and uric acid) have peak concentrations within a 24-hour period after an individual is subjected to stress (Najem et al., 1997). Thus stress (work related or otherwise) is a risk factor for stone formation.

1.2.5 Age

In children under the age of 15, urinary stone disease shows an initial peak during the first two years which reappears at puberty (Reis-Santos, 2000). Bladder stones in children are caused by a nutritionally poor diet that is low in animal protein, calcium, and phosphate, but high in cereal and is acidogenic (Robertson, 2003).

Data from epidemiological studies as well as studies based on hospital admittance figures have demonstrated that there is a marked increase in stone disease in adults between the second and fifth decade of life (Ljunghall et al., 1975; Robertson et al., 1979; Ljunghall, 1987; Reis-Santos, 2000). Recently, some authors have shown a third peak at about 60 years.

That advancing age may be a contributing factor to stone disease was examined in a recent study by Gergsland et al. (2002) who found that the urine of healthy young persons (under 20 years old) vigorously inhibited the growth of calcium oxalate crystals *in vitro*. Changes in serum concentrations of calcium and creatinine with increasing age have indicated clinical and metabolic disturbances that may enhance the risk of stone formation (Siener et al., 2000). Renal development from birth to adulthood and the concomitant increase in renal concentrating capacity has been shown to increase the risk of crystallization in the loop of Henle. This coincides with the increasing incidence of calcium oxalate urolithiasis (Kok et al., 1999). It is possible that differences in renal architecture also exit between blacks and whites in South Africa.

1.2.6 Race and Sex

Idiopathic stone disease occurs more frequently in white caucasians than in blacks, irrespective of the geographic area (Ramello et al., 2000). Brazil has reported a 4-to-1 caucasian to black ratio between stone formers. In the USA, kidney stone disease is more prevalent in whites than blacks (Soucie et al., 1994). Interestingly, the prevalence of nephrolithiasis in black Americans has significantly increased since their conversion to caucasian diet (Ramello et al., 2000).

In South Africa, kidney stones occur in 15 % of the white population, whereas it affects less than 1 % of the dominant black population (Meyers et al., 1994). The reason for this vast difference in the incidence of renal calculi has yet to be ascertained. Much research has gone into investigating urine composition in the two race groups, but results have not identified differences which might explain the low stone incidence in blacks (Whalley et al., 1998; Whalley et al., 1999; Meyers et al., 1994). Dietary differences between the two race groups have also been studied (Lewandowski et al., 2001). It has been suggested that the absence of stones in blacks could be due to differences in renal mechanisms for handling oxalate in the two race groups (Lewandowski et al., 2001), or a disparity in the presence or absence of promoters and inhibitors of renal stone formation (Whalley et al., 1998). Notwithstanding some interesting results, the mystery of the black population's low stone incidence has not been resolved. However, attention has recently been focussed on the potentially superior inhibitory role of urinary proteins in South African black subjects (Craig et al., 2000; Durrbaum et al., 2000; Craig et al., 2001; Durrbaum et al., 2001; Webber et al., 2003). This is discussed further in paragraphs 1.5.1 and 1.5.4

Regarding gender, men have both higher stone incidence rates and higher mean urinary oxalate concentrations than women (Curhan, 1999). Several investigators have attributed the lower incidence of nephrolithiasis in females to their excretion of larger amounts on urinary citrate (Shorr et al., 1942, Hodgkinson et al., 1962; Welshman et al., 1976; Menon et al., 1983, Parks et al., 1986; Hammar et al., 1987; Trinchieri et al., 1992). Sex hormones have also been shown to be involved in the pathogenesis of calcification: androgens appear to increase and estrogens appear to decrease urinary oxalate excretion and kidney calcium oxalate deposition (Fan et al., 1999; Iguchi et al., 1999; Iguchi et al., 2000; Heller et al., 2002).

1.3 States of Supersaturation

The physicochemical theory of stone formation seeks to explain it in terms of the physical chemistry of supersaturated solutions, and in terms of the effects of various inhibitors of crystal nucleation, growth and aggregation (Rose, 1982).

1.3.1 The Fundamental Role of Supersaturation

The thermodynamic driving force for the change of phase from solution to solid (crystallisation) or the reverse (dissolution) is supersaturation. Supersaturation represents the excess of free energy between the two phases (Hess et al., 1996; Kavanagh, 2000). It is expressed as the difference in chemical potential of the solution (μ_{soln}) and the chemical potential of the crystalline phase at equilibrium (μ_{cryst}). If μ_{soln} - μ_{cryst} is positive, crystallisation occurs and if the difference is negative dissolution will occur. The chemical potentials can be expressed in terms of the gas constant (R), the absolute temperature (T) and the activities of the solution (a) and the solution at equilibrium (a_{eq}) (Hess et al., 1996; Kavanagh, 2000).

$$\Delta G = \mu_{soln} - \mu_{cryst} = RT\ln(a/a_{eq}) \qquad (1)$$

The fundamental dimensionless driving force for crystallisation is

$$\Delta G = \frac{\mu_{soln} - \mu_{cryst}}{RT} = \ln(a/a_{eq}) = \ln S \qquad (2)$$

S is the dimensionless supersaturation, also called the supersaturation ratio. When S<1 the solution is undersaturated. When S=1 it is saturated and when S>1 it is supersaturated. Another term used to express the level of saturation is the *relative* supersaturation (σ), where $\sigma = S - 1$. Supersaturated solutions are those with $\sigma > 0$ (Hess et al., 1996; Kavanagh, 2000).

1.3.2 Thermodynamics of Crystallisation

Stone formation is a crystallisation process in supersaturated urine (Hess and Kok, 1996). Urinary supersaturation with respect to stone minerals is the driving force for the formation of crystal nidus (nucleation) and their transformation to a visible particle (crystal growth) (Hess and Kok, 1996; Baumann et al., 1997). The extent of supersaturation affects the kinetics of nucleation, growth, ageing and aggregation which in turn determine crystal size distribution (Kavanagh, 2000). Because of the extremely low solubility of calcium oxalate, even normal urine is always supersaturated with respect to this salt (Nordin et al.,

1993). As such, excretion of small crystals in urine occurs both in healthy individuals and in kidney stone formers (Hess et al., 1996; Fleisch, 1978). Thus, the precipitation of calcium oxalate in urine by itself is not a pathological problem as long as the particles formed are allowed to pass freely through the urinary tract. Problems occur only if the particles are retained and allowed to form the nidus of a stone (Kok et al., 1994).

Nucleation

Urine falls into one of three zones of relative supersaturation (Fig 3, modified from Robertson and Nordin, 1973). Urine below the solubility product (S<1) of a given mineral is undersaturated with respect to that mineral. Any crystals of the mineral added to it will dissolve.

Urine lying between the solubility product and the formation product (S>1) of homogeneous nucleation, as illustrated in fig 3, is defined as being metastable (Nordin et al., 1993). Urine within the metastable zone may exist for long periods without precipitation taking place spontaneously (Robertson et al., 1982). The closer the urine is to the formation product of homogeneous nucleation, the shorter is the latent period before crystallisation begins. For a given level of metastable supersaturation, this lag-time may be shortened by the presence of nucleating material (such as cell debris, certain macromolecules or crystals of other stone-forming salts) in the urine. This process is called heterogeneous nucleation (Hess and Kok, 1996).

Finally, urine which attains or exceeds the formation product of homogeneous nucleation is said to be in the labile or unstable zone in which rapid, prolific nucleation takes place (Nordin et al., 1993). Homogeneous nucleation rarely occurs in urine due to the presence of many foreign surfaces for heterogeneous nucleation of crystals (Hess and Kok, 1996). Urinary supersaturation can be calculated by a special computer program (EQUIL), which is based on the chemical analysis of 23 urinary components and the calculation of 103 complexes (Brown et al., 1994).

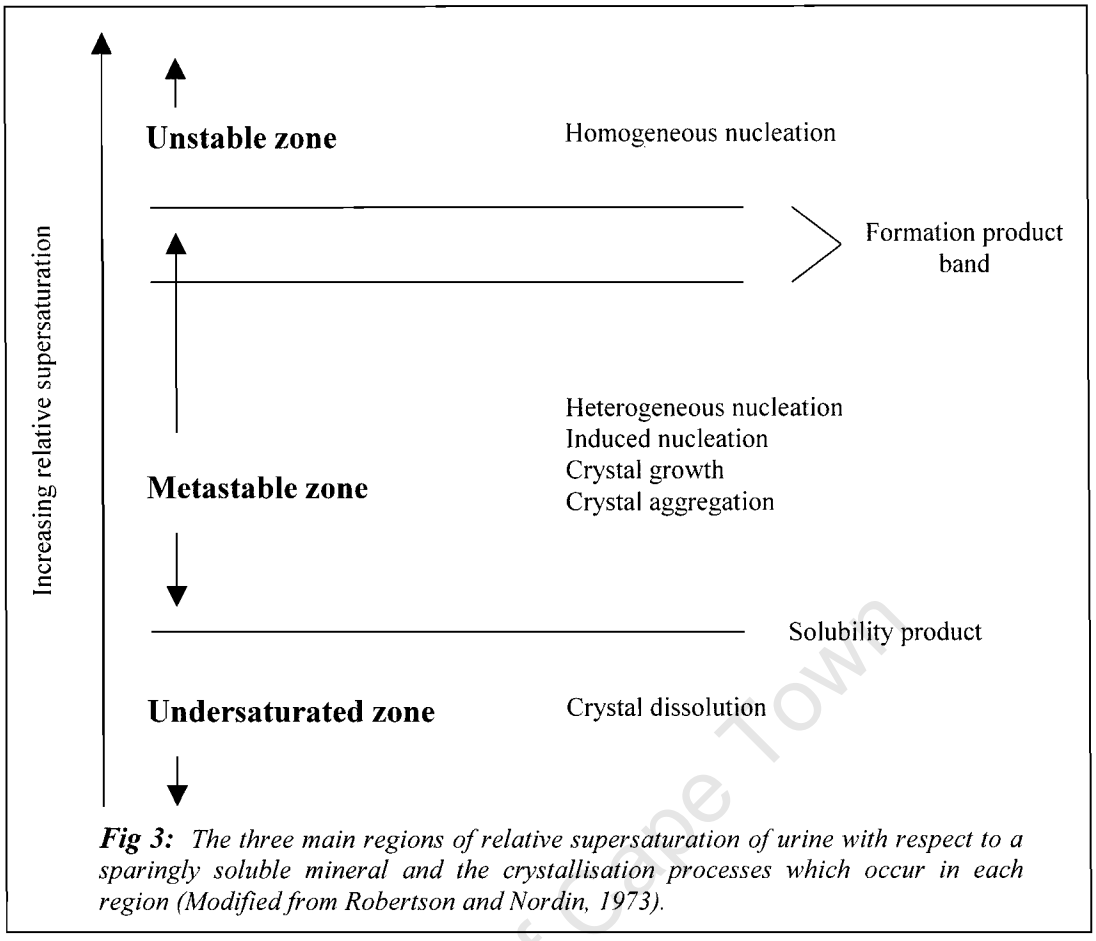


Fig 3: *The three main regions of relative supersaturation of urine with respect to a sparingly soluble mineral and the crystallisation processes which occur in each region (Modified from Robertson and Nordin, 1973).*

There are two phenomena that are closely related to heterogeneous nucleation – secondary nucleation and epitaxy. Secondary nucleation refers to the nucleation of new crystals on pre-existing surfaces of their own species, as described above. Epitaxy is the process whereby material of one crystal type is precipitated upon the surface of another whose lattice dimensions are almost identical (Hess and Kok, 1996). For example, stones from recurrent calcium stone-formers contain a larger fraction of calcium phosphate than those from single stone-formers. Thus, the calcium oxalate – calcium phosphate epitaxy appears to be of clinical significance (Hess and Kok, 1996).

Growth

Crystal growth is the addition of new crystal components onto a crystal nucleus (Hess and Kok, 1996). The following differential equation, taken from the review by Gill (1984), can be applied to the kinetics of crystallisation:

$$-\frac{dS}{dt} = \frac{dP}{dt} = \square AS^{\eta} \qquad (3)$$

where S = supersaturation, t = time, P = precipitate, □ = reaction rate constant, A = surface area for crystallisation, η = order of the reaction. For calcium oxalate at low to medium supersaturations in the metastable range, $\eta = 2$. Thus integration of equation (3) becomes

$$-\frac{1}{s} = \square At - \frac{1}{S_o} \qquad (4)$$

where S_o = initial supersaturation. By keeping the seed crystal surface area constant in metastable supersaturated solutions, the rate constant □ can be derived with and without added inhibitor substances which can then be utilised in the Langmuir adsorption isotherm plot to compare inhibitory activities (Gill, 1984). The rate of calcium oxalate crystallisation in whole urine can be measured directly by adding specific quantities of calcium oxalate seed crystals and measuring changes in either ^{14}C or ^{45}Ca in the supernatant and/or precipitated fractions (Gill, 1984).

Crystal growth in aqueous solution can take place via speciation, bulk diffusion and adsorption. Speciation is the binding of monomers to form complexes. Monomers migrate through the bulk solution by a process called bulk diffusion and attach to the crystal surface by adsorption (Hess and Kok, 1996).

Aggregation

Aggregation is the process whereby crystals in solution stick together in order to form a larger particle. Crystal aggregation is energetically favoured and thus a natural process. It can occur at all states of saturation - undersaturated, saturated and supersaturated conditions (Hess and Kok, 1996).

Crystal aggregation is considered to be more important for stone formation than crystal nucleation and growth. The reason for this, as pointed out in the review by Hess and Kok (1996), is that calcium oxalate growth is too slow to produce clinically significant particles within the transit time in the urinary tract, whereas aggregation occurs within seconds and is thus more dangerous for the formation of large crystalline particles in renal tubules. Also, ultrastructural analyses of kidney stones reveal a highly aggregated structure (Hess and Kok, 1996).

1.3.3 Mechanism of Stone Formation

Crystallisation in the urinary tract occurs via three mechanisms – nucleation, growth and/or aggregation (Gill, 1984; Hess and Kok, 1996). Nucleation is the transformation of solution ions into the solid phase. Normal urine is virtually always supersaturated with respect to calcium oxalate (Nordin et al., 1993). Yet, supersaturation with respect to calcium oxalate rarely reaches levels required for spontaneous nucleation (or homogeneous nucleation) of calcium oxalate crystals. However, crystals may nucleate at lower levels of supersaturation in the presence of nucleating sites, a process referred to as heterogeneous nucleation (Kavanagh, 2000). Such sites may be provided by cell membranes, proteins or by crystals of other types such as apatite and brushite. Heterogeneous nucleation of calcium oxalate can be provoked by such stimuli *in vitro*, and is thought to be a frequent event in the process of stone formation, as stones often consist of mixtures of more than one type of crystal, as well as membrane fragments and a mixture of proteins (Hess and Kok, 1996; Kavanagh, 2000). Unless crystals are retained in the urinary tract, nucleation will not lead to stone formation. Crystalluria occurs in normal subjects, but these are washed out harmlessly in the urine (Worcester, 1996). If enough nuclei are formed and continue to grow unhindered, they will aggregate into larger particles which have a greater probability of becoming trapped in the kidney (Worcester, 1996).

Alternatively, the individual nuclei and aggregates may adhere to membrane walls or be deposited within the kidney and continue to grow and aggregate as the supersaturated urine flows past until they are too large to pass the kidney (Meyer, 1990).

1.4 Naturally Occurring Urinary Inhibitors

Human urine normally accommodates considerable supersaturations, with multiple materials, yet the incidence of renal stones worldwide is less than 1 % (Coe et al., 1991). The main reason why stone disease does not occur in all persons is that normal urine retards and inhibits crystallisation (Pak et al., 1976). This observation initiated what came to be known as the inhibitor theory of stone formation which states that stones result from a deficiency or lack of urinary inhibitors of crystallisation (Ryall, 1997). It has provided both a possible explanation for the occurrence of stones and a theoretical basis for its prevention. If stones form because urine lacks some inhibitor that normally prevents crystallisation, further episodes can be prevented by correcting the deficiency with medication that has the same inhibitory effects (Ryall, 1997).

According to Finlayson et al. (1978), for a substance to be considered as an inhibitor, it should hinder the aggregation of individual crystals into larger crystal masses which may become large enough to lodge in renal tubules and form the nidus of a stone. Alternatively, crystals may bind to specific sites on renal epithelial cells, leading to retention and eventual growth into renal stones (Lieske et al., 1992). An inhibitor should interfere with this process and thus diminish the possibility that a crystal will set in motion the chain of events that will eventually result in a stone (Worcester, 1996). Also, by adsorbing onto the crystal surface, an inhibitor interferes with the formation of the crystal lattice and retards the attachment of new ions, thus inhibiting nucleation, and most importantly, growth and aggregation into larger crystals, which otherwise would form in supersaturated urine (Marangella et al, 2000).

Some of the protein inhibitors are produced within the nephron, by a process that can be triggered or enhanced by the crystals themselves (Marangella et al., 2000). Therefore, an increase in their intra-tubular concentration may be seen as a defence mechanism of the kidney against lithogenesis. Anionic inhibitors, by adsorbing onto the crystal surface or modifying the crystal structure can prevent adhesion to fixed anionic sites on the tubular cells (Marangella et al., 2000). Based on composition, inhibitors are subdivided into micromolecular inhibitors and macromolecular ones; the latter group includes glycosaminoglycans (heparin, hyaluronic acid and chondroitin sulphate) and proteins (Marangella et al., 2000).

1.4.1 Micromolecular Inhibitors

Pyrophosphate

Pyrophosphate occurs in human urine at a concentration of 4 mg/*l* (March et al., 2001). It has been reported to inhibit hydroxyapatite precipitation in synthetic urine at concentrations of 2.87 x 10^{-6} M (Grases et al., 2000). Studies have been conducted to investigate the effect of pyrophosphate on calcium oxalate crystallisation. It is believed to bind to calcium in the solid phase rather than in the solution phase. It also seems to bind to calcium oxalate monohydrate and not to the dihydrate (Shirane et al., 1993). This observation is of significance to stone disease because in 70 % of upper urinary tract calculi, calcium oxalate monohydrate forms the central core (Gill et al., 1974; Gill, 1984). Results for and against the inhibitory effect of pyrophosphate in whole human urine abound (Hallson et al., 1983; Ryall et al., 1985; Sidhu et al., 1986; Baumann et al., 1989)

It has been reported that pyrophosphate from stone formers has a reduced inhibitory effect on calcium oxalate crystallisation (Conte et al., 1989). This was attributed to failure of the renal transformation of orthophosphate into pyrophosphate (Conte et al., 1989).

Clinical studies involving the administration of neutral potassium phosphate salt (UroPhos-K) to stone patients showed that it reduced the urinary saturation of calcium oxalate. In addition, by increasing urinary excretion of inhibitors (citrate and pyrophosphate), it reduced the propensity for spontaneous nucleation of brushite (the formation product of brushite increased) and inhibited crystal aggregation of calcium oxalate (Breslau et al., 1995).

Citrate

Citrate inhibits calcium oxalate crystal nucleation (Doremus et al., 1978), growth (Meyer et al., 1975) and aggregation (Ryall et al., 1981) in aqueous media and inhibits calcium oxalate deposition in undiluted (Ryall et al., 1985) and concentrated (Hallson et al., 1983) urine. Although citrate's inhibitory effect on crystal nucleation and growth can be attributed to its complex formation with calcium, it is also known to bind to the calcium

oxalate crystal surface, a property that might explain its influence on both crystal growth and aggregation. Thus, citrate is both a chelator and crystal poison (Ryall, 1997).

Based on the above findings, it is not surprising that low urinary citrate excretion (hypocitraturia) is an accepted pathogenic factor for calcium renal stone formation (Hosking, 1985; Pak, 1991). There are several factors that have been found to modulate the urinary excretion rate of citrate. Ingestion of excess animal protein leads to a decrease in urinary pH, which in turn lowers urinary citrate (Kok et al., 1990; Pak, 1991).

Both scientific and clinical findings indicate that citrate excretion is a significant determinant of stone formation, and citrate supplementation has become an accepted form of stone therapy (Ryall, 1997). Indeed, several citrate-containing preparations have been successfully used in the management of calcium oxalate nephrolithiasis. These include potassium citrate (Pak et al., 1985; Pak et al., 1986; Whalley et al., 1996), sodium potassium citrate (Schwille et al., 1985; Schwille et al., 1987; Ogawa, 1994), calcium citrate (Sakhaee et al., 1994; Levine et al., 1994), calcium-sodium citrate (Schwille, 1997), potassium-magnesium citrate (Pak et al., 1992; Ettinger et al., 1997) and sodium citrate bicarbonate (Allie et al., 2003).

Magnesium

Just like citrate, magnesium has been found to form soluble complexes with oxalate. It has been shown to reduce calcium oxalate crystal formation in concentrated human urine (Hallson et al., 1982). This effect was attributed to competition by magnesium with calcium for the oxalate ion, leading to the formation of the more soluble magnesium oxalate (Hallson et al., 1982). It is for this reason that magnesium has been labelled an inhibitor of calcium oxalate crystallisation (Desmars et al., 1973).

Oral administration of magnesium has been found to be successful in the prophylactic treatment of urinary stones. In a two year study by Johansson et al. (1980), patients with renal stone disease were given a prophylactic treatment containing magnesium hydroxide and thereafter the mean stone episode rate monitored and compared to a group of patients who only received general advice and no prophylactic treatment. Of the 56 patients who received prophylactic treatment over a minimum of two years, 45 remained free of

recurrences or continued stone growth (total recurrence rate of 12 %). The mean stone episode rate during treatment was 0.03 stones per year compared to 0.22 stones per year before treatment was instituted. Of the 34 patients who received no prophylactic therapy, 15 had experienced recurrences after 2 years (44 % recurrence rate). Therefore, in comparison, treatment with magnesium hydroxide appeared to reduce the recurrence rate. Apart from minor gastrointestinal discomfort no adverse effects were observed during treatment (Johansson et al., 1980).

A recent study (Siener et al., 1995), has reported that the influence of a magnesium-rich vegetarian diet on urinary magnesium concentration and excretion is not as high as expected. The oxalic acid content of the plant foods resulted in hyperoxaluria. Stone formers are therefore advised to avoid an excess of fruits, vegetables and cereals. These observations are in harmony with previous studies that have demonstrated that a balanced diet coupled with adequate fluid intake is effective in reducing the risk of calcium oxalate stone formation (Hesse et al., 1993; Goldfarb, 1994; Parivar et al., 1996; Giannini et al., 1999; Massey et al., 2001).

1.4.2 Macromolecular Inhibitors

Kidney stones only develop in the presence of an organic matrix which forms a precisely structured framework of the stones. This matrix constitutes on average 1-3 % of the stone weight but is spread throughout the entire stone structure (Morse et al., 1988). The association of macromolecules with stones raises the possibility that they play a role in their formation (Jones et al., 1990; Ryall, 2000). These macromolecules are essentially glycosaminoglycans GAGs and proteins (Boyce, 1968; Morse et al., 1988; Ryall et al., 2000).

Glycosaminoglycans (GAGs)

Since it was discovered that GAGs - heparin, chondroitin sulphate (ChS) and hyaluronic acid (HA) – affected the precipitation of calcium oxalate (Crawford et al., 1968), several tests have been carried out to determine the effects of natural and synthetic GAGs on calcium oxalate crystallisation (Wabner et al., 1991; Yamaguchi et al., 1993; Shum et al., 1993; Shirane et al., 1995; Senthil et al., 1996; Chan et al., 1998). The only GAGs that

have been shown to be present in stones are heparan sulphate (Roberts et al., 1986), and hyaluronic acid (Yamaguchi et al., 1993). Heparan sulphate (HS) has been shown to inhibit aggregation of calcium oxalate crystals in inorganic medium (Shirane et al., 1989), enhance calcium oxalate crystal nucleation and inhibit growth in frozen urine (Shum et al., 1993). However, it does not affect the urinary metastable limit with respect to calcium oxalate or mass deposition of the salt in undiluted, ultrafiltered urine (Suzuki et al., 1996). Nevertheless, these findings for HS suggest that it may play a role in inhibiting calcium oxalate crystallisation. ChS, the most prominent urinary GAG (Roberts, 1986) has not been detected in stones and heparin does not exist in human urine (Ryall, 1997).

Proteins

Proteins are a highly diversified class of biomolecules. Differences in their chemical properties, such as charge, organic functional groups, shape, size and solubility enable them to perform many biological functions. These functions include enzyme catalysis, metabolic regulation, binding and transport of small molecules, gene regulation, immunological defence and cell structure (Stryer, 1995).

Proteins whose inhibitory effects on calcium oxalate crystallisation have been determined in either inorganic reaction solutions or undiluted urine include albumin (Edyvane et al., 1986; Ryall et al., 1991; Dussol et al., 1995; Grover et al., 1998), Tamm-Horsfall glycoprotein - THG (Hess et al., 1989; Hess, 1992; Hess, 1994), uropontin (Shiraga et al., 1992; Worcester, 1992; Worcester et al., 1995; Worcester et al., 1996), nephrocalcin (Nakagawa et al., 1983; Coe et al., 1991; Chang et al., 2001), inter α-trypsin inhibitor (Sorenson et al., 1990; Atmani et al., 1993; Dawson et al., 1998; Moriyama et al., 2001) and urinary prothrombin fragment 1 or UPTF1 (Ryall et al., 1995; Ryall et al., 1997; Durrbaum et al., 2000; Webber et al., 2003). Besides THG which can inhibit or promote calcium oxalate crystallisation depending on experimental conditions (Grover et al., 1990; Hess, 1992; Hess, 1994), the other proteins listed above have been shown to inhibit the nucleation, growth or aggregation of calcium oxalate crystals, and have accordingly been credited with performing some function in the formation of calcium oxalate stones (Grover et al., 1998). However, because the effects of these proteins have been tested individually using different techniques, it is impossible to assess their relative contributions to the crystallisation of calcium oxalate *in vivo*, and consequently their possible influence on

stone formation (Grover et al., 1998). The proteins mentioned above will be discussed in detail in the section below.

1.5 Urinary Proteins and Kidney Stones

The Organic Matrix

What researchers in stone disease know for sure about the organic matrix is that it is a universal component of all human renal calculi which accounts for some 2-5 % by weight of most stones (Boyce et al., 1956). An in-depth study of the structure of the organic matrix of human urinary concretions by Boyce (1968) showed that it is a heterogeneous material composed of 64 % protein, 9 % non-amino sugars, 5 % glucosamine, 10 % bound water and 12 % organic ash (Boyce, 1968). According to this author, the adsorption of macromolecules onto the surface of crystals is a selective process that is necessary for stone formation. As such, matrix deposition may be the differentiating factor between stone formers and healthy people. The fact that the matrix is largely composed of different proteins is an indication that they play some role in stone pathogenesis. What though is their source? Nucleation and early growth of calcium oxalate crystals within renal tubules results in the incorporation of normal urinary organic components (Finlayson et al., 1961; Khan et al., 1987). This is the first type of matrix. Once the crystal aggregate becomes lodged in the renal tubule, trauma resulting from abrasion of the urothelial lining will change the macromolecular composition of the urine bathing the crystals and will result in the deposition of a second type of matrix which is chemically different from that occluded in crystals during nucleation and early growth (Doyle et al., 1991). Evidence supporting this is the presence in stones of serum proteins which are too large for glomerular filtration (Boyce et al., 1962), lipids – a very nominal component of urine (Khan et al., 1988), erythrocytes, mitochondrial ghosts and bacteria (Finlayson et al., 1984).

Proteins detected in calcium oxalate stones include Tamm-Horsfall glycoprotein (THG), albumin, α- and γ-globulins, haemoglobin, neutrophil elastase, transferrin, α_1-microglobulin, CD59 protein (protectin), superoxide dismutase, α_1-antitrypsin, uropontin (osteopontin), nephrocalcin, β_2-microglobulin, α_1-acid glycoprotein, renal lithostathine, urinary prothrombin fragment 1 (UPTF1) and inter α-trypsin inhibitor (Ryall, 1996). One

approach to identifying possible functions for proteins in stone formation is to examine their individual effects on calcium oxalate crystallisation. This has been attempted for relatively few stone proteins. The next few paragraphs will discuss results obtained thus far on THG, nephrocalcin, uropontin (osteopontin), urinary prothrombin fragment 1 (UPTF1), uronic acid-rich protein (inter-α-trypsin inhibitor, bikunin) and albumin.

1.5.1 Tamm-Horsfall Glycoprotein (THG)

THG is an acidic urinary glycoprotein with a monomeric molecular weight of 80 000 daltons or 80 kDa (Ryall, 1997) that is synthesised and secreted by epithelial cells in the thick ascending limb of the loop of Henle, and early distal convoluted tubule (Kumar et al., 1990). 20-200 mg of protein is excreted in urine each day (Hunt et al., 1994). THG has been found to exhibit different effects on calcium oxalate crystallisation depending on ambient conditions and methodology. Under conditions of high ionic strength and low pH, it self-aggregates (Hess et al., 1989; Hess, 1992; Hess, 1994). Elevated concentrations of THG itself also favour self-aggregation. The state of aggregation influences whether THG will inhibit or promote crystallisation (McQueen et al., 1966; Hess, 1994). THG only has a minor effect on crystal growth (Hess, 1994). It has little effect on calcium oxalate nucleation, although it appears to promote crystal nucleation at very high ionic strength and low pH (Hess, 1994). All the same, it is a potent inhibitor of calcium oxalate crystal aggregation in undiluted, ultrafiltered urine (Ryall et al., 1991). This property results from steric hindrance and not from adsorption onto the crystal surface (Ryall et al., 1991). A study by Hess and associates (1989) using a spectrophotometric assay demonstrated that THG is a strong inhibitor of crystal aggregation in a concentration-dependent manner, inhibiting aggregation by 50 % at concentrations of 10^{-8} mol/*l* and nearly 90 % at 5×10^{-8} mol/*l*. However, increases in the ionic strength of the assay solution (from, 0.1 to 0.28) or decreases in pH (from 7.2 to 5.7) greatly diminished the inhibitory activity of THG. This decrease in inhibitory activity was directly related to an increase in viscosity of THG in solution caused by self-aggregation (Hess et al., 1989). Thus, conditions of ionic strength and pH that may be seen in urine can lead to highly polymerised THG that fails to inhibit crystal aggregation and may even promote aggregation in certain situations (Worcester, 1996).

Recently, THG has been isolated from the urine of South Africa's stone free black and stone prone white populations and has been tested in various crystallisation experiments (Craig et al., 2000; Craig et al., 2001). Several interesting results have been found. THG isolated from blacks (BTHG) was found to be a more potent inhibitor of COM crystal aggregation than that from whites (WTHG) (Craig et al., 2001). It was therefore speculated that perhaps the relative immunity of the black population to calcium oxalate stone formation might be linked to the more potent activity of this race group's THG in inhibiting aggregation. Various techniques have been used to characterise the biochemical properties of THG from normal and stone-forming black and white South Africans. These include matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF), amino acid analysis and tryptic finger printing digestion (Craig et al., 2000). No compositional or structural differences were found (Craig et al., 2000).

1.5.2 Nephrocalcin

Nephrocalcin (NC) is produced in the epithelium of the proximal tubules and thick ascending limp of the loop of Henle (Nakagawa et al., 1990). It has a monomeric molecular weight of 14 kDa, although the protein can self-associate into larger aggregates (Nakagawa et al., 1990; Mustafi et al., 1996; Worcester, 1996). NC contains γ-carboxyglutaminc acid or Gla (glycosylated) and considerable numbers of phosphate residues (phosphorylated), therefore its net charge is strongly anionic (Coe et al., 1991). Nakagawa et al (1985) reported that NC has the ability to form stable films at air-water interfaces with a high collapse pressure. They attributed this to NC's amphiphilic structure – it has hydrophilic and hydrophobic regions. This feature may depend on the presence of Gla (Coe et al., 1991; Chang et al., 2001).

Hydrophilic regions would possess an affinity for the charged crystal surface, whereas the hydrophobic surface would be oriented toward the solution, offering solution ions a poor surface for lodging, thus discouraging overgrowths of new crystals over the protein coating (Coe et al., 1991). This asymmetry is a crucial property of an inhibitor because as these authors pointed out, in a radially symmetrical inhibitor, there will be a fixed and stable array of charged sites on all sides – like the bristles on a cylindrical hair brush. The charged regions that attach to the crystals would be mirrored on the solution side by an exact replica that solution ions would recognise and bind to just as the protein bound to the

crystal, offering to ions an ideal surface for heterogeneous nucleation. Kidney tubules provide a constant flow of new ions, so crystals at a collecting duct, for example would be constantly bathed by a solution with ions that are constantly being replenished, and could grow at leisure if not covered by a film that inhibited growth (Coe et al., 1991).

In vitro experiments show that NC can inhibit the growth of calcium oxalate crystals in metastably supersaturated calcium oxalate solutions (Nakagawa et al., 1983; Chang et al., 2001). The addition of increasing amounts of NC to the solution causes progressive growth inhibition. Growth inhibition requires NC concentrations of 10^{-6} to 10^{-8} mol/l, similar to those found in urine (Nakagawa et al., 1983). NC's ability to inhibit growth is related to its ability to bind to a crystal's surface, where it blocks growth sites in the crystal lattice structure (Nakagawa et al., 1983). When calcium oxalate crystals are incubated in solutions of NC and adherence of the protein to crystal surface is measured with anti-NC antibody, directly measured adsorption increases in parallel with inhibition (Worcester et al., 1988). Other data (Deganello, 1991) suggest that NC binds in a preferential manner to certain crystal faces and blocks growth of the crystal along those planes. NC can also inhibit calcium oxalate crystal nucleation (Asplin et al., 1991). When a few large well-formed crystals of calcium oxalate were incubated in a metastably supersaturated solution of calcium oxalate, nucleation of new crystals occurred at the crystal surface, whereas growth of the original crystals accounted for only a negligible amount of loss of solute from the solution (Asplin et al., 1991). Addition of 5×10^{-7} mol/l NC to the solution prevented formation of new crystals. Thus, concentrations of NC similar to those found in urine can prevent calcium oxalate crystal nucleation in vitro (Asplin et al., 1991). NC has been found to exert inhibitory effects on calcium oxalate crystal aggregation. Aggregation inhibition was studied in vitro (Hess et al., 1989) using a spectrophotometric assay in which calcium oxalate crystals were suspended in a solution that was just saturated with respect to calcium and oxalate. NC was found to be a potent aggregation inhibitor at all concentrations above 5×10^{-7} mol/l. At that concentration, aggregation was inhibited by 80% (Hess et al., 1989). NC isolated from urine of calcium stone formers has been found to have structural abnormalities, which reduce growth inhibition efficiency and amphiphilicity (Nakagawa, 1997). The defects in stone formers possibly arises from a single primary sequence abnormality that prevents proper golgi apparatus processing for Gla, and also affects calcium binding sites needed to form crystal surface attachment (Coe et al., 1991).

1.5.3 Uropontin (Osteopontin)

Uropontin has been isolated from human urine and has been found to inhibit calcium oxalate crystal growth (Worcester, 1992). Its N-terminal sequence is identical to that of osteopontin, a protein that is found in bone matrix, produced by osteoblasts (Butler, 1989). The polypeptide backbone has a molecular weight of 32 kDa. The bone-derived and renally derived forms appear to be very similar with respect to amino acid sequence, and are presumably products of the same gene, but may differ in their post-translational modifications, such as glycosylation, phosporylation and sulphation (Negata et al., 1989; Hoyer, 1994). It is rich in aspartic acid, and has a conserved sequence of 8-10 aspartic acid residues in the N-terminal portion of the protein (Worcester, 1996). Uropontin (osteopontin) can inhibit both spontaneous nucleation (Worcester et al., 1995), growth (Worcester et al., 1992) and aggregation (Asplin et al., 1995) of calcium oxalate crystals in supersaturated solutions.

When crystals nucleate in supersaturated solutions of calcium oxalate in the absence of the protein, the crystal habit produced is calcium oxalate monohydrate (COM). However, in urine the crystals found are predominantly calcium oxalate dihydrate (COD) (Elliot et al., 1980). This is especially true in normal subjects; in stone formers with crystalluria, COM crystals are seen with increased frequency (Elliot et al., 1980). Addition of several inhibitory macromolecules to supersaturated solutions, including polyaspartic acid and osteopontin purified from cell culture, promotes the formation of COD in preference to COM (Wesson et al., 1995). This may be of significance because additional studies suggest that COD crystals have a lower affinity for renal papillary cell membranes than do COM crystals (Wesson et al., 1995). Thus, another role of urinary inhibitors such as uropontin might be to favour formation of COD crystals over COM in supersaturated solutions, which might be less likely to bind to renal epithelial cells and be retained in the kidney (Worcester; 1996). Indeed, it has recently been demonstrated (Lieske et al., 1995) that uropontin (osteopontin) inhibits the adhesion of COM crystals to renal epithelial cells in culture.

1.5.4 Urinary Prothrombin Fragment 1 (UPTF1)

Studies of calcium oxalate crystals freshly precipitated in urine, revealed a protein present in significant quantities but which could not be detected in normal urine (Doyle et al., 1991). Amino acid sequencing revealed that this protein had N-terminal homology with human prothrombin (Stapleton et al., 1993). Further study showed that the protein was a urinary form of the F1 activation peptide of prothrombin and it was therefore called urinary prothrombin fragment 1 (UPTF1). It is a glycoprotein with a molecular mass of 31 kDa (Stapleton et al., 1995). It is located specifically in the human kidney in the thick ascending limb of the loops of Henle and the distal convoluted tubules, where it is present in greater quantities in stone formers compared to healthy controls (Stapleton et al., 1993).

UPTF1 is a potent inhibitor of both calcium oxalate crystal growth and aggregation in undiluted, ultrafiltered human urine (Ryall et al., 1995), a property that is attributed to its ten Gla residues located in the N-terminal region of the molecule (Ryall, 1997). It has therefore been suggested that variations in the amount of the protein excreted in urine, or alterations in its molecular structure, may possibly predispose some individuals to urolithiasis (Ryall, 1997). However, more investigation is required to confirm the role of this protein in providing protection from stone formation.

Like THG, UPTF1 has recently been investigated in the black and white populations in South Africa (Durrbaum et al., 2000; Durrbaum et al., 2001; Webber et al., 2003). Here too, important results have been found. The *in vitro* inhibitory effects of UPTF1 isolated from urine of healthy black (BF1) and white (WF1) subjects have been studied on calcium oxalate crystallisation using a "cross-over" design in which each protein was tested for its effect in its own endogenous urine as well as in the urine obtained from the other race group (Durrbaum et al., 2001). BF1 was found to be a superior inhibitor compared to WF1, but more interestingly, the urine from black subjects induced a more powerful inhibitory effect from both proteins relative to urine from white subjects (Durrbaum et al., 2001). Biochemical characterisation of UPTF1 from the two race groups has shown no differences in molecular weight or amino acid sequence and composition (Durrbaum et al., 2001).

In another study, the amount of UPTF1 trapped in crystals formed in urine from the two races was compared (Webber et al., 2003). Crystals from the black and white groups' control samples were composed mainly of calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) respectively. Pure COM and COD were prepared from both race groups' urine by adjustment of the calcium concentration to 0.5 and 12 mM respectively. A significant amount of UPTF1 was included in COM with undetectable amounts in COD crystals. The black group's COM crystals were found to contain more UPTF1 per milligram of calcium oxalate than those of the white group. The authors concluded that the composition of black subjects' urine is more favourable for UPTF1's binding than urine from whites. It was therefore inferred that the greater amount of intracrystalline UPTF1 from the black group may promote the dismantling of crystals by the action of urinary proteases and thus play a protective role (Webber et al., 2003).

1.5.5 Uronic-Acid-Rich Protein (Inter-α-trypsin-Inhibitor, Bikunin)

Inter-α-trypsin-inhibitor (ITI) was first isolated in urine in 1990 by Sorenson and colleagues, and was found to be an inhibitor of calcium oxalate growth in inorganic medium (Sorenson et al., 1990). It comprises three protein chains linked by chondroitin sulphate: two heavy chains H1 and H2 and a light chain most commonly known as bikunin. The physiological function of the two heavy chains is unknown; neither have they been found in urine (Dawson et al., 1998). The protein, derived from the urine of stone formers has been shown to have a reduced inhibitory effect on calcium oxalate crystallisation as compared to that of healthy subjects (Atmani et al., 1994).

A similar protein has been isolated in rat urine (Atmani et al., 1995). Like its human counterpart, it cross-reacted with anti-ITI, this suggested that it belonged to the ITI super family, as does a 35 kDa urinary protein (Tang et al., 1995) with N-terminal sequence identical to bikunin, the light chain of ITI. Evidence suggests that all of these proteins are one and the same – bikunin. This supposition is supported by the recent demonstration that uronic-acid-rich protein is actually bikunin (Atmani et al., 1996).

Bikunin inhibits calcium oxalate crystal nucleation and growth (Atmani et al., 1999) in inorganic systems and is present in calcium oxalate crystals precipitated from urine (Atmani et al., 1996). It has been found to inhibit COM crystal adhesion to renal tubular

cells (Ebisono et al., 1999). It may therefore contribute to the regulation of crystal adhesion and retention within tubules during stone formation (Ebisono et al., 1999). Hyperoxaluria and renal calcium oxalate crystal deposition in rat models result in the increased expression of crystallisation inhibitors, such as inter-alpha-inhibitor related proteins (Moriyama et al., 2001). Thus, the kidneys respond to nephrolithic challenges by producing proteins, such as bikunin, that inhibit crystal formation and retention (Moriyama et al., 2001).

Bikunin isolated from urine of renal stone formers is de-glycosylated and has an inferior inhibitory effect on calcium oxalate crystal growth compared to protein from healthy subjects (Suzuki et al., 2001). Also, since stone formers produce a higher concentration of the protein, it would appear that its effect on the pathogenesis of urolithiasis is linked to its concentration and degree of glycosylation.

1.5.6 Albumin

After THG, albumin is the most abundant protein in urine (Grover et al., 1998). The presence of albumin in the organic matrix of kidney stones suggests that it may play a role in nephrolithiasis (Morse et al., 1988).

A matrix profile was recently obtained by SDS gel electrophoresis of the pooled EDTA extracts of five different kidney stones (Dussol et al., 1995). It featured albumin as the major component of the stone matrix and proposed that albumin is the protein that binds most other proteins to form the matrix of all stones. This study had some severe limitations which were pointed out by Binnette and co-workers (1996). Firstly, in order to gather enough material to study, pooled stone extracts were used, thus excluding the possibility of detecting individual variations in composition. Also, by using SDS gel electrophoresis they may have failed to detect differences in protein composition which could have been revealed by 2-D gel electrophoresis. In addition, no mention was made of a residue after extraction. Previously, researchers had discovered that dialysis against EDTA-containing solutions removed most of the crystallising component of the stones, leaving a residue which proved difficult to solubilise. Finally, proteins considered by other investigators (Grover et al., 1998) as major components of matrix were not reported (Binette et al., 1996).

The effects of albumin on calcium oxalate crystallisation in inorganic solution (Hess; 1989), undiluted ultrafiltered urine (Edyvane et al., 1987; Ryall et al., 1991) as well as in whole urine (Edyvane et al., 1987; Cerini 1999) have been determined. Many researchers have reported that it is an inhibitor of calcium oxalate aggregation (Edyvane et al., 1987; Worcester, 1996; Grover, 1998). It has also been shown that albumin reduces the size of crystal aggregates and causes a small increase in the amount of crystal matter precipitated (Edyvane et al., 1987; Ryall et al., 1991). Albumin inhibits calcium oxalate monohydrate aggregation detectably at 0.01 μM which is near its normal urinary concentration (Hess et al., 1989). The reduction in size of crystal aggregates caused by albumin has been measured at 32 % of the initial value – 5.9nm versus 8.6nm (Grover et al., 1998). At concentrations of 0.005 % to 0.10 % (v/v), it has no effect on the metastable limit of urine as detected by Coulter Counter (Edyvane et al., 1987). However, it caused a marked increase in the number and volume of calcium oxalate crystals deposited in response to an oxalate load. Analysis of particle size distributions showed that the crystals deposited in the presence of albumin were smaller than those occurring in the control. This apparent promotion effect is due to its inhibition of crystal aggregation which increases the crystal surface area available for calcium oxalate deposition (Edyvane et al., 1987).

Albumin's effect on calcium oxalate growth has been described as modest or absent depending on experimental conditions. In a dilute seeded crystallisation system, albumin was found to be a weak inhibitor of crystal growth (Edyvane et al., 1987). Worcester et al. (1988) reported that albumin has a strong affinity for COM crystals, yet did not inhibit crystal growth at even 3×10^{-6} M. They therefore proposed that either almost all of the adsorption occurred at non-growth sites or the protein adsorbed to growth sites but did not prevent growth (Worcester et al., 1988). Similar results were obtained by Grover and associates (1998) who found that crystal growth, as measured by [^{14}C]-oxalate disappearance was unaffected by HSA. Other investigators have found HSA to display significant affinity for COM crystals (Worcester et al., 1988; Dussol et al., 1995).

Edyvane et al. (1987) noted that albumin increased the volume and number of calcium oxalate crystals precipitated from whole urine. They also reported that the crystals deposited in the presence of albumin were smaller than those occurring in the control. In a more recent study by Cerini et al. (1999) it was demonstrated that albumin purified by immunoaffinity chromatography from the urine of healthy subjects was a more potent

nucleator of calcium oxalate than commercial serum albumin. Nucleation was evaluated by the number of crystals generated from a metastable calcium oxalate solution in polystyrene wells coated with albumin which had been derived from healthy subjects and idiopathic calcium stone formers. Also, calcium oxalate crystals induced by albumin from healthy subjects was significantly more numerous than albumin from idiopathic calcium stone formers. Cerini and co-workers have pointed out that, at first sight, promotion of nucleation could be considered unfavourable because one would assume that keeping the number of calcium oxalate crystals formed in primitive urine to a minimum would facilitate the control of their growth by specific inhibitors. However, they argued that increasing the number of crystal nuclei would be beneficial because it would decrease calcium oxalate saturation, thus limiting the size that crystals could reach and favouring their elimination by urine flow. The fact that healthy subjects have significantly smaller crystals in their urine than stone formers supports this (Cerini et al., 1999).

Prompted by the above observation, Chen et al. (2001) investigated the overall effect of albumin on calcium oxalate crystallisation. At a concentration of 100nM albumin was found to promote nucleation by 8.9 % relative to the control. Inhibition of crystal growth was determined using the spectrophotometric assay developed by Asplin and associates (1997). Albumin exerted an inhibitory effect on crystal growth, with IC_{50} obtained from regression analysis being 37.5 nM. Finally, at a concentration of 100 nM, albumin exerted an inhibitory effect of 72.73 % on crystal aggregation. Thus, the overall effect of albumin on calcium oxalate crystallisation may be as an inhibitor (Chen et al., 2001). Their results were compatible with those of Cerini and co-workers mentioned above.

Another approach to establishing the association of proteins with crystals is to study them in calcium oxalate crystals freshly precipitated from healthy human urine since they will not contain proteins that are released secondarily due to tissue damage (Finlayson et al., 1961; Khan et al., 1987; Morse et al., 1988; Morse et al., 1989; Doyle et al., 1991). A major finding of these studies is that inclusion of proteins into crystals is a selective phenomenon (Doyle et al., 1991; Doyle et al., 1995). However, THG and albumin, the most abundant proteins found in urine were not detected in the crystal matrix. It was demonstrated that the major protein associated with urinary crystals is UPTF1 (Doyle et al., 1991; Ryall, 1997). Nevertheless, in these earlier studies, human urine samples were centrifuged and filtered before inducing crystallisation. Atmani et al. (1996) later

discovered that these procedures contributed to the loss of a significant portion of the urinary macromolecules and that experiments should be conducted in whole human urine without prior centrifugation and filtration. Another significant outcome of their study was that not only prothromnin-related proteins but also albumin and particularly osteopontin are associated with calcium oxalate crystals formed in human urine provided the crystals are washed only with distilled water and not with NaOH. Perhaps these proteins were not tightly bound to the crystals and were removed easily by washing with a strong agent such as NaOH. They proposed that the presence of these proteins in crystal matrix may not be as selective as previously reported. Their presence may indicate that they are involved in modulating crystallisation either indirectly or in concert with each other to give a resultant effect (Atmani et al., 1996).

1.6 Objectives

The presence of stone free and stone prone populations in South Africa presents a unique research opportunity to investigate the pathogenesis of this disease. The kidney stone research group at the University of Cape Town (of which the present investigator is a member), has recently been studying the role of urinary proteins in this context. Indeed, results for Tamm-Horsfall protein and urinary prothrombin fragment 1 have already been described earlier in this chapter. The project described in this thesis undertook to extend these investigations to the urinary protein, albumin. This protein was selected on the basis of its reported inhibition of calcium oxalate aggregation which is widely regarded as the key crystallisation mechanism in kidney stone formation.

The objectives of this study were therefore:

(1) to isolate and purify albumin from the urine of healthy black and healthy white male subjects;

(2) to test and compare the effects of the protein from each race group, as well as that of commercial human serum albumin on the kinetics of calcium oxalate crystallisation;

(3) to measure the zeta potentials of calcium oxalate monohydrate crystal slurries which have been coated with these proteins;

(4) to measure the degree of aggregation inhibition effected by these proteins on calcium oxalate monohydrate crystal slurries in sedimentation experiments;

(5) to test and compare the effect of these proteins on the volume-size distribution of calcium oxalate crystals in *in vitro* crystallisation experiments;

(6) to test and compare the effects of these proteins on calcium oxalate crystal deposition rates using labelled [^{14}C]-oxalate.

CHAPTER 2: ISOLATION, PURIFICATION AND VERIFICATION OF URINARY ALBUMIN

2.1 Introduction

Albumin is one of the most abundant proteins in urine. Normal levels of urinary albumin are approximately 5 - 30 mg/*l* for a 24 hr urine sample (Ruhn et al., 1994). Using concentrated urine samples, Cerini and co-workers (1999) reported that, on average, albumin excretion in healthy subjects is about 2.40 mg/24hr. However, the distribution in individuals covered a wide range (0.192 to 21.8 mg/24hr) (Cerini et al., 1999). This observation has been confirmed by other investigators and is due to the state of hydration of the body. The volume of urine excreted can be highly variable depending mainly on the individual's fluid intake and physical activity. In dilute urine, the total protein excretion may be underestimated. If the urine is concentrated, as frequently occurs after strenuous physical activity, an increased protein concentration could be misinterpreted (Newman et al., 2000).

Several different approaches have been used for the isolation of urinary albumin. These include diethylaminoethyl (DEAE)-cellulose column chromatography (Hess et al., 1989); high-performance liquid chromatography (Dussol et al., 1995); immunoaffinity chromatography (Cerini et al., 1999); and high-performance immunoaffinity chromatography and flow injection analysis (Ruhn et al., 1994). These methods rely on the pre-treatment of urine. In the present study two approaches of sample preparation were used.

The first method of sample preparation used was developed by Cerini et al. (1999) and describes the preparation of concentrated urine samples. Briefly, urine samples were ultrafiltered using an Amicon hollow fibre bundle (H1P 10) with a nominal cut-off of 10 kDa relative molecular mass. The concentrate was extensively dialysed in distilled water until most of the pigment was removed, and then lyophilised. Lyophilised samples were resuspended in PBS and albumin isolated from this mixture by immunoaffinity chromatography.

The second method of sample preparation used was to grow calcium oxalate crystals in whole urine as described by Atmani et al. (2002), and then demineralise the crystals in EDTA. Inorganic impurities were then removed by dialysis. This method yielded a pigment free protein extract from which albumin was isolated by immunoaffinity chromatography.

Immunoaffinity chromatography was selected for the isolation of albumin in this study because previous work had shown that it is a relatively quick and efficient approach (Cerini et al., 1999). It is a molecular technique which can be used to isolate antibodies, antigens, hormones or other proteins by taking advantage of their binding affinity for their respective ligands. The desired protein is isolated from a crude extract through its specific binding to an antibody that has been coupled to a gel matrix. By changing column conditions, the analyte can be eluted for quantification or collected for further use (Ruhn et al., 1994). The procedure can be divided into 3 steps as illustrated in Fig 4.

The first step involves the binding of the ligand (antibody) to the sepharose matrix to form a ligand-matrix complex, which is packed into a column. Next, a crude extract is passed through the column and the ligand's recognized substrate binds to the ligand-matrix complex, halting its passage through the gel. Unbound impurities are removed from the gel column using a strong wash of high ionic strength. Finally, a stronger more acidic second wash is used to elute the desired protein. This second wash relies on the reversible binding properties of the ligand, which allows the bound protein to dissociate from its ligand in the presence of this stronger wash (Ruhn et al., 1994).

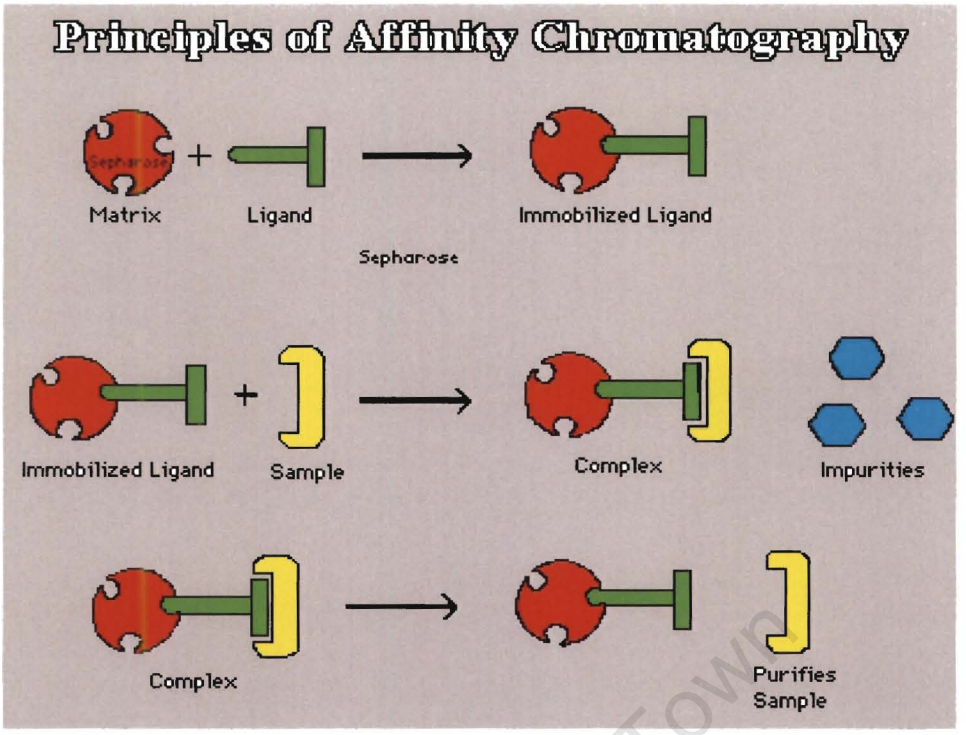


Fig 4: *Steps for affinity chromatography.* ***Top panel:*** *bonding of ligand to sepharose matrix results in immobilised ligand-matrix complex.* ***Middle panel:*** *sample mixture is added to gel column. Complimentary proteins bind to ligand-matrix complex, while the non-binding impurities are washed out.* ***Bottom panel:*** *to remove the bound proteins and retrieve them from the gel, a stronger second wash is run through the column and the desired protein is washed out. (Figure taken from http://ntri.tamuk.edu/fplc/pursammat.html).*

In this study two different matrices for the isolation of urinary albumin were used. The first matrix was CNBr-activated sepharose to which anti-human albumin (anti-HA) was coupled directly. This has the advantage of being very specific and having low background. The disadvantage is lower binding capacity, as the orientation of the antibody coupled to the CNBr matrix is not optimal for albumin binding, because coupling of the antibody to the matrix is through free amine groups. Repeated use of the column also leads to loss of antibody from the column.

To circumvent this problem, a second approach was employed in which anti-HA was cross-linked to protein-G sepharose beads. Protein-G is a cell wall protein of certain pathogenic bacteria which specifically binds to the Fc region of the immunoglobulin or antibody (Sjobring et al., 1989). This ensures that both binding sites on the immunoglobulin are available to bind to albumin (Fig 5). Thus, protein-G sepharose has a much better binding efficiency since it orientates the anti-HA for optimal binding to human serum albumin (HSA).

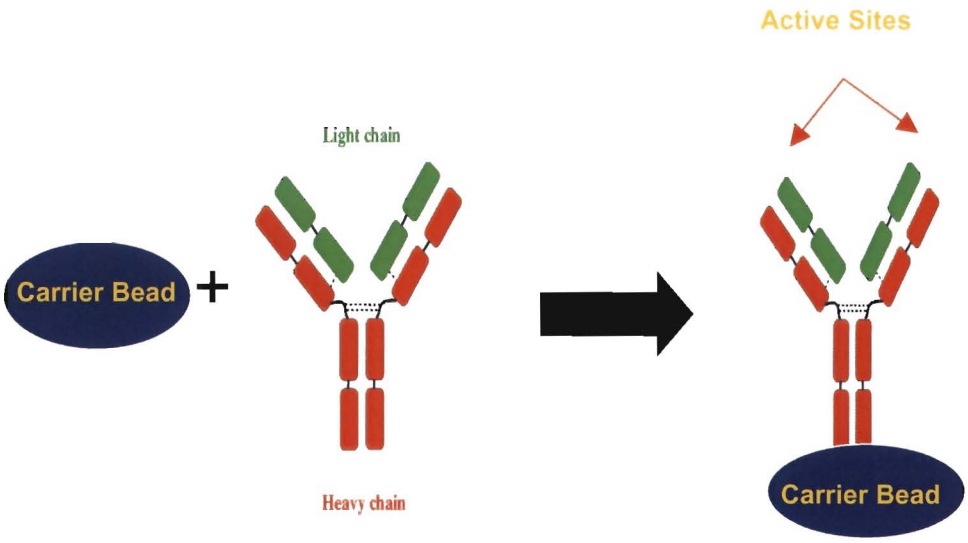


Fig 5: *Mechanism of binding of Protein-G to an antibody. (Figure modified from www.bch.bris.ac.uk/staff/henerson/Immunoglobulins2.htm)*

2.2 Method

2.2.1 Urine Collection and Sample Preparation

Preparation of Concentrated Urine Samples

24 hour urine samples were collected from 20 healthy black and 20 healthy white subjects in 2 *l* plastic bottles containing 10.0 g of boric acid, to prevent calcium oxalate precipitation. The absence of blood and infection from individual urine samples was confirmed using urinalysis test strips (Combur 10 test strips; Boehringer Mannheim). Individual urine samples were filtered with a pre-filter (Macherey-Nagel), followed by a 0.420 μm Millipore filter (Millipore Corporation, Bedford), and then pooled. Pooled samples from each race group were ultra-filtered using an Amicon hollow fibre bundle (H1P 10) with a nominal cut-off at 10 kDa relative molecular mass. The concentrate was dialysed extensively for two days in distilled water at 4 ^{o}C with two changes of 20 *l* of distilled water for every 500 ml of concentrated urine. The dialysate was then lyophilised. Lyophilised samples from black and white subjects were resuspended in 280 ml and 240 ml of PBS respectively. Albumin was isolated from these samples using the CNBr – anti-HA sepharose column.

Preparation of Crystal Matrix Extract

CaOx crystallisation

Preparation of urine for the isolation of albumin by protein-G sepharose was carried out as follows. 24-hour urine specimens were collected from 2 healthy black and 7 healthy white male subjects and tested for blood and infection as described above, and then pooled. (The number of white subjects used was greater than the number of black subjects because CaOx crystals induced in urines from the former group were required for the optimisation experiments described in the demineralisation paragraph below). An adapted method of that reported by Atmani et al. (2002) was used for the induction of calcium oxalate crystallisation in whole urine. Pooled urines from the race groups were warmed to 37.0 °C in a shaking water-bath. CaOx crystallisation was induced by drop-wise addition of 15.0 ml/*l* of 100 mM sodium oxalate solution at pH 5.9 - 6.5. After 1 hour, the same amount of sodium oxalate was added to the mixture. An hour later, the crystals were harvested by centrifugation at 8000 *g* using a JR-20 rotor (Beckman Instruments, Fullerton, Calif. USA) for 30 min at room temperature.

Demineralisation

Preliminary experiments demonstrated that the volume of EDTA used in demineralising the CaOx crystals had an effect on the amount of dissolved albumin. It was therefore necessary to optimise the demineralisation process by dissolving 1.14 g of crystals formed in urine from white subjects, in different volumes of EDTA. The optimum volume was found to be 10.0 ml.

Crystals formed in urine from black and white subjects were demineralised in 10.0 ml 250 mM EDTA, pH 8, per gram of crystals for three days with continuous stirring at 4 °C. The supernatants, BCME (crystal matrix extract from blacks) and WCME (crystal matrix extract from whites), were dialysed in distilled water for 24 hours at 4 °C, then lyophilised and resuspended in distilled water (100 µ*l* of water per 10.0 mg of extract). BCME and WCME were then analysed by 10 % SDS-PAGE and Western blotting.

2.2.2 Isolation of Albumin from Concentrated Urine Using a CNBr - anti-HA Sepharose Column

Coupling of anti-HA to CNBr activated sepharose

2.86 g CNBr sepharose beads (Sigma) were activated by suspension in 600 ml ice-cold 1 mM HCl, washed for 15 min and filtered using a sintered glass funnel. 1.00 ml of a 21.9 mg/ml anti-HA (Sigma) solution was added to 25.0 ml of 100 mM $NaHCO_3$ in 500 mM NaCl, pH 8.3. The beads were transferred into the solution containing the antibody and coupled overnight at 4 °C. Following filtration, the beads were resuspended in 50.0 ml of 1.00 M ethanolamine, pH 8, and rotated for 2 hours to block free binding sites. Excess adsorbed protein was washed away using 250 ml 100 mM $NaHCO_3$ in 500 mM NaCl, pH 8.30, followed by 500 ml 100 mM sodium acetate buffer in 500 mM NaCl, pH 4.00, and followed by 250 ml 100 mM $NaHCO_3$ in 500 mM NaCl, pH 8.30. The beads were then transferred to a 10.0 ml column. The column's binding efficiency was tested with 20.0 ml 0.1 mg/ml solution of commercial HSA.

Purification of albumin from concentrated urine

Samples from black and white subjects, previously equilibrated in PBS (section 2.2.1), were centrifuged at 3939 *g* for 15 min, and then loaded onto the column at a flow rate of 0.400 ml/min. The column was subsequently washed thoroughly with PBS. A more stringent wash followed using 1 M NaCl in PBS. Albumin was eluted with 100 mM glycine, pH 2.80 and neutralised by the addition of a few drops of 1.00 M Tris, pH 9.50. The fractions containing the protein were pooled and dialysed extensively against distilled water at 4 °C. Protein concentration was measured using the Bradford protein assay (Bradford, 1976).

2.2.3 Albumin Isolation from Crystal Matrix Extract Using a Protein G – Anti-HA Sepharose Column

Coupling and cross-linking of anti-HA to Protein-G Sepharose

0.4 g protein-G sepharose was washed three times in 10.0 ml PBS, pH 7.50, then transferred to 10.0 ml of a 7.65 mg/ml solution of anti-HA (Sigma) in PBS and rotated overnight at 4 °C. The mixture was centrifuged and the beads washed three times in 10.0 ml PBS. Cross-linking of bound antibody to protein-G involved resuspending the beads in 3.00 M NaCl. 50.0 mM borate buffer, pH 9.00 and incubating for one hour in 20.0 mM dimethylpimelimidate (DMP). This was followed by washing with 200 mM ethanolamine, pH 8.00, resuspension in ethanolamine and incubation at room temperature for 2 hours. Finally, the mixture was centrifuged for one minute, washed with PBS and transferred into a 2 ml column.

Purification of albumin from crystal matrix extract

Crystal matrix extract from each race group, resuspended in distilled water, was passed through the column at a flow rate of 0.4 ml/min, and then washed overnight with PBS to ensure the removal of any unbound material. As before (paragraph 2.2.2), albumin fractions were eluted with 100 mM glycine, pH 2.80 and neutralised by the addition of a few drops of 1.00 M Tris, pH 9.50. Fractions were pooled and dialysed overnight against distilled water at 4 °C. The Bradford assay was used to determine the protein concentration in the dialysate (Bradford, 1976).

2.2.4 Bradford Protein Concentration Determination

The Bradford method was followed to measure protein concentration (Bradford, 1976). Standard solutions of bovine serum albumin (BSA) were prepared as specified by the manufacturers (BioRad Laboratories, Hercules, Calif. USA). $OD_{595\ nm}$ readings were taken against a reagent blank and used to plot a protein standard curve from which protein concentrations were determined.

2.2.5 SDS Polyacrylamide Gel Electrophoresis

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated on a 10 % Polyacrylamide mini-slab gel using the BioRad Mini-Protean II apparatus (BioRad Laboratories, Hercules, Calif. USA). The samples as well as a low molecular weight marker (LMM) were dissolved in sample application buffer and boiled for five minutes. The gel was run at 25.0 mA constant current for one hour. Protein bands were stained with Coomassie brilliant blue, or transferred onto nitrocellulose membrane (Amersham Life Science, Germany) for immunological identification by Western blotting. (Please refer to appendix for SDS-PAGE solutions).

2.2.6 Western Blotting

Proteins were immunodetected by Western blotting. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane at 100 V for one hour at room temperature using a transfer buffer consisting of 150 mM glycine, 20 % methanol and 20.0 mM Tris, pH 8.30.

The nitrocellulose membrane was incubated for an hour in blocking buffer containing 20.0 mM NaCl, 5 % skim milk, 0.1 % Tween-20 and 50.0 mM Tris, pH 7.40. The membrane was subsequently immersed in anti-HA (Sigma), the primary antibody, at a 1:1000 dilution in blocking buffer for 90 min and then washed six times, each wash being a minute long, with blocking buffer before incubation in the secondary antibody – Peroxidase-labelled affinity purified antibody (KPL Europe, Guildford, UK) – at a dilution of 1:2000 in blocking buffer for 60 min, then washed six times again in blocking buffer.

The developer contained 24.0 mg 4-chloro-1-naphthol in 8.00 ml ice cold methanol, 40.0 ml Tris buffered saline and 20 μl H_2O_2. The bands were allowed to develop in darkness until the desired exposure had been reached. Then the developer was poured off and the membrane rinsed in distilled water.

2.3 Results

2.3.1 Optimisation of Demineralisation

Protein extracts from calcium oxalate crystals that were demineralised in 10.0 ml and 100 ml of EDTA were analysed by 10 % SDS-PAGE and Western-blotting (Fig 6).

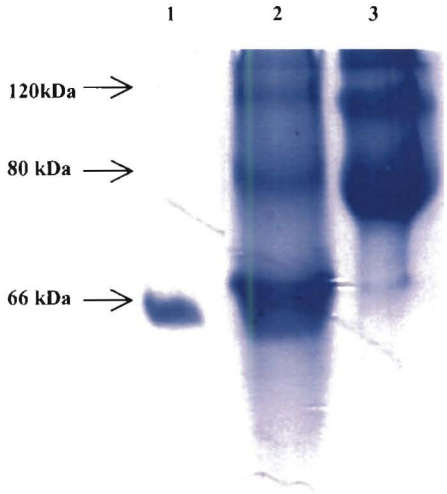


Fig 6.1

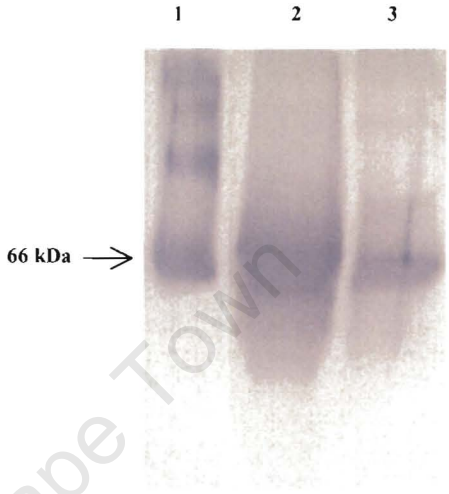


Fig 6.2

Fig 6: *10 % SDS-PAGE (Fig 6.1) and Western blot (Fig 6.2) of proteins obtained from demineralised calcium oxalate crystals induced in urine from white subjects (WCME). Lane 1 is commercial HSA, lane 2 and 3 correspond to WCME that was demineralised in 10.0 ml and 100 ml EDTA respectively.*

Crystals demineralised in 10.0 ml EDTA gave a prominent band at 66 kDa (Fig 6.1, lane 2), which the Western blot confirmed was albumin (Fig 6.2, lane 2). Demineralised crystals in 100 ml EDTA, gave prominent bands at 80 and 100 kDa and only a minor band at 66 kDa. The band at 80 kDa is likely Tamm-Horsfall mucoprotein (THM) (Ryall, 1997). Thus the optimum volume for demineralization was found to be 10ml. Earlier work by Atmani and co-workers (2002) has demonstrated that different proteins can be associated with newly formed crystals in urine and that these proteins do not all bind to the crystals in the same way. Albumin for example is an intermolecular protein, unlike UPTF1 which is included in crystals, as such it requires only small volumes of EDTA for demineralisation.

2.3.2 Immunoaffinity Chromatography Using CNBr-Activated Sepharose

Anti-HA coupled to CNBr-activated sepharose beads was used to purify urinary albumin. Of the 21.9 mg anti-HA present in the coupling solution, 2.81 mg was present in the filtrate after coupling. Thus, 87.2 % of anti-HSA had successfully been coupled to the CNBr-activated sepharose beads.

To test the column's binding efficiency, 20 ml 0.1 mg/ml solution of commercial HSA was applied to the column. Fig 7 shows the elution profile that was obtained.

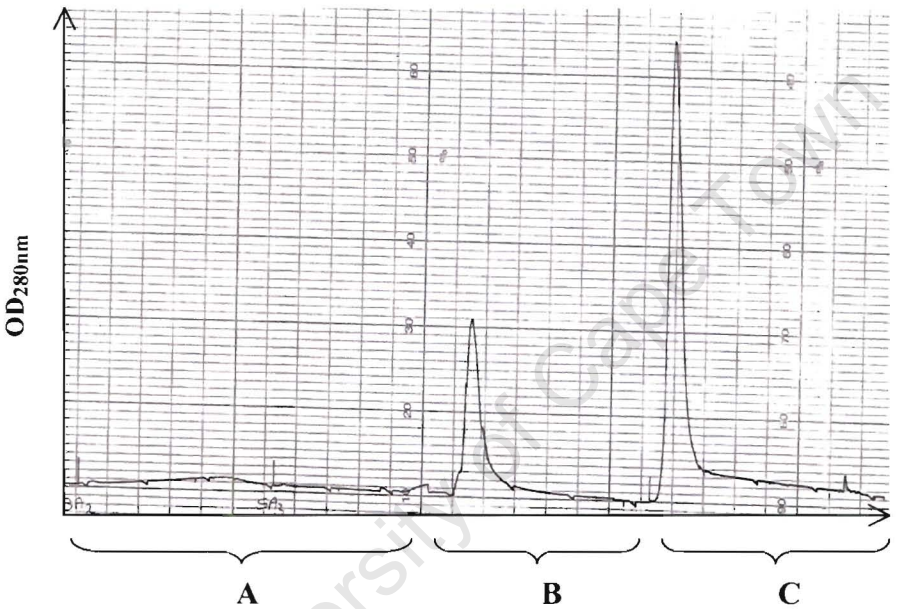


Fig 7: *Elution profile for 0.1 mg/ml HSA on the CNBr-activated sepharose column as determined by UV absorbance at 280 nm. 2.00 mg HSA in 20.0 ml PBS (A) was loaded onto the column at a flow rate of 0.4 ml/min, the column was then washed with 15.0 ml 1M NaCl in PBS (B), pH 7.50. The protein peak was eluted with 12.0 ml 100 mM glycine (C), pH 2.8.*

The sample application eluent contained 87.5 μg HSA (4.40 %; Fig 7A), indicating that not all the albumin was binding to the column. The salt wash had 118 μg HSA (8.90 %; Fig 7B) and the glycine wash contained 929 μg of HSA (46.5 %; Fig 7C). It is evident that the binding efficiency of the column is low. Even though 19.1 mg of anti-HA had successfully been bound to the CNBr matrix, the recovery of HSA was only 46.5 %.

The elution profile (Fig 7) showed that albumin had successfully been bound by the antibody, since no peak was obtained during sample application. It seemed though that the salt solution used was strong enough to cause some of the bound albumin to leach off the

column. However, most of the protein remained bound until it was eluted with 100 mM glycine, pH 2.80.

Albumin from 600 ml and 3.60 *l* of urine from black and white subjects, respectively, was purified using the CNBr - anti-HA affinity column. 0.368 mg and 1.17 mg of purified albumin was isolated from blacks and whites respectively. This translates to 0.613 mg/*l* and 0.325 mg/*l* of protein in urine from black and white subjects respectively. The purity of the isolated protein was analysed by 10 % SDS-PAGE and a single band at 66 kDa corresponding to albumin was observed in both BA and WA (Fig 8).

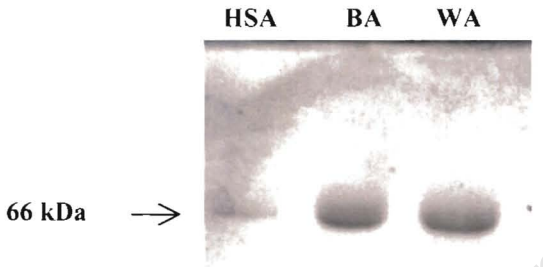


Fig 8: *10 % SDS-PAGE of the isolated proteins. Lane 1: 2.00 µg HSA, lane 2: 4.00 µg BA, lane 3: 4.00 µg WA. Coomassie brilliant blue was used to stain the protein bands.*

At this point, it was noted that the glycine peak was progressively decreasing with each applied sample. The column was therefore re-tested with 20 ml 0.1 mg/ml solution of HSA. Most of the protein was eluted during sample application and salt wash (profile not shown) suggesting that the anti-HA had leached off the column. Since the column was no longer binding albumin, a new column was prepared using protein-G sepharose, which binds to anti-HA in such a way that both binding sites on the antibody are exposed for maximum binding to albumin.

2.3.3 Immunoaffinity Chromatography Using Protein-G Sepharose

The purity of anti-HSA was checked by 10 % SDS-PAGE before using it to prepare the protein-G sepharose column (Fig 9).

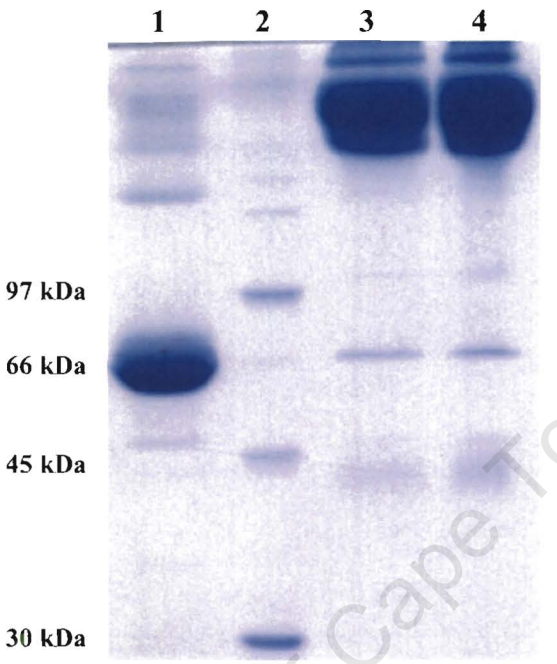


Fig 9: *Non-reducing 10 % SDS-PAGE used to analyse the purity of anti-HA. Lane 1: 10.0 µg HSA, lane 2: low molecular weight marker, lane 3: 10.0 µg anti-HA, lane 4: 8.00 µg anti-HA.*

No mercaptoethanol was added to the samples containing the antibody. Immunoglobulins have 4 polypeptide chains. Two identical heavy chains (H) and 2 identical light chains (L). H and L are linked by disulfide bonds. Mercaptoethanol is a reducing agent that breaks up disulphide bonds. Since no mercaptoethanol was added, the H and L chains did not dissociate and a high molecular weight band was observed for the antibody as indicated in Fig 9. There is a very faint band at 66 kDa in the anti-HA lane, indicating the presence of trace amounts of albumin. However, as indicated in the next section, the column was thoroughly washed so this band was absent from future gels. The SDS gel showed that anti-HA had not degraded and was suitable for the preparation of the Protein-G sepharose column.

Of the 76.5mg anti-HA present in the coupling solution, 21.7 mg was retained in the filtrate after coupling as determined using the Bio-Rad assay. 54.8 mg (71.5 %) of anti-HSA had successfully been coupled to the protein-G sepharose beads.

Column Binding Experiments

Protein-G sepharose was coupled to anti-HA as described previously. The column was equilibrated with 20.0 ml PBS. To test whether the cross-linking of the antibody to protein-G sepharose was successful, the column was washed with 10.0 ml 100 mM glycine at pH 2.80. The pH was readjusted to 7.40 with 20.0 ml PBS. This sequence (glycine wash at pH 2.80 followed by PBS wash at pH 7.40) was repeated once more. Since no peak was obtained from the second glycine wash it was concluded that the antibody was not leaching off the column (profile not shown).

14.6 mg HSA was loaded onto the column, washed with PBS and then eluted with 100 mM glycine, pH 2.80 (profile not shown). The salt wash was excluded from this and all subsequent runs because experiments on the CNBr-activated column had shown significant quantities of HSA were eluted when this step was included.

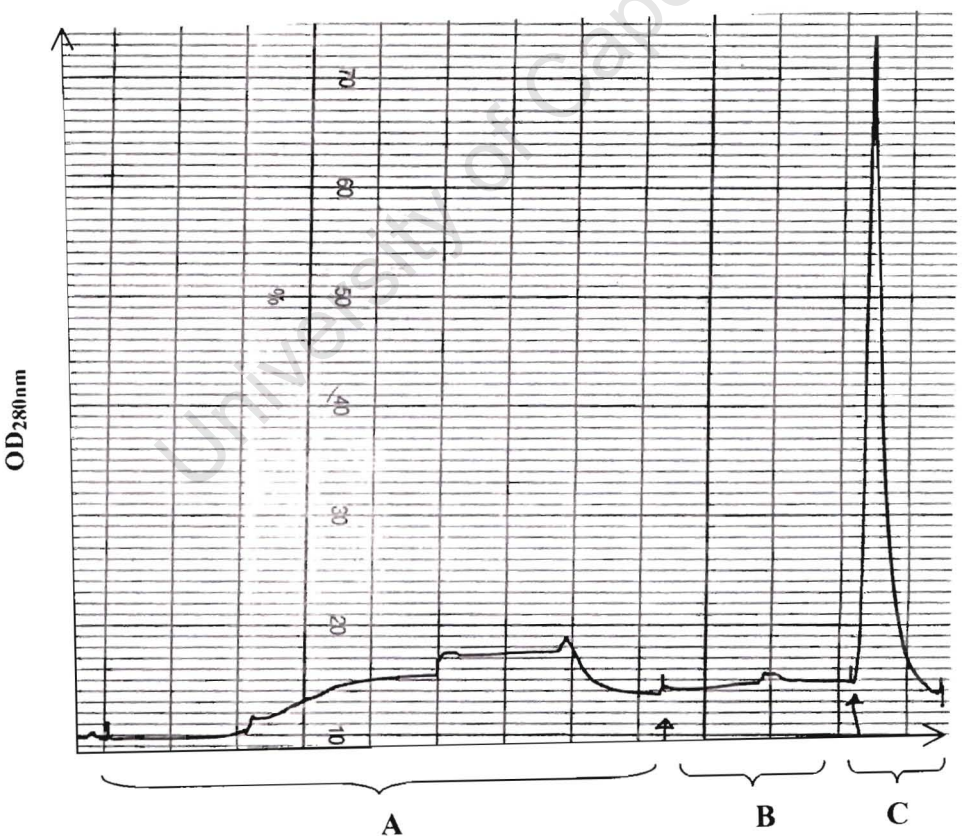


Fig 10: *Elution Profile for 0.234 mg/ml HSA on Protein-G Sepharose Column as determined by UV absorbance at 280 nm. 11.7 mg HSA in 50.0 ml PBS (A) was applied to the column at a flow rate of 0.4 ml/min. It was subsequently washed with 20.0 ml (B) PBS and 10.0 ml 100 mM glycine (C), pH 2.80.*

Having primed the column with HSA, its binding efficiency was tested with 50.0 ml of a 0.234 mg/ml solution of HSA, loaded at a flow rate of 0.4 ml/min. The elution profile confirmed that the antibody was binding to albumin (Fig 10). 6.76 mg of protein was obtained in the sample application eluent. 0.031 mg and 3.99 mg HSA was recovered in the PBS wash and glycine eluent respectively. Of the 11.7 mg HSA that was applied to the column, about 4 mg was recovered (i.e. only 34 % bound to the column).

10.0 μg of protein from each of the eluents in Fig 10 was loaded onto a 10 % polyacrylamide gel and stained with coomassie brilliant blue. Each sample had a prominent band at 66.0 kDa which corresponds to HSA (Fig 11).

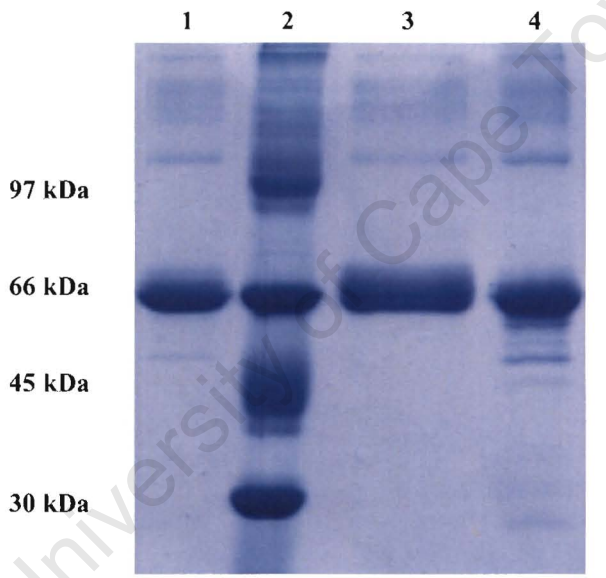


Fig11: *10 % SDS-PAGE was used to analyse 10.0 µg of protein from peak A and C (in fig 11 above) and their mobility compared to that of commercial HSA (that had not been loaded onto the column). Lane1: commercial HSA (not from column), lane 2: low molecular weight marker, lane 3: peak A, lane 4: peak C.*

Having established the binding efficiency of the column 129 ml BCME (prepared from 1.82 *l* urine from black subjects) and 247 ml WCME (prepared from 6.61 *l* urine from white subjects) was applied and HSA eluted as described above. After dialysis, 1.46 mg and 4.91 mg of albumin was recovered from BCME and WCME respectively. This translates to 0.800 mg/*l* and 0.740 mg/*l* of protein in urine from black and white subjects respectively. The protein yield was four-fold higher than the yield obtained using the CNBr column. Thus the protein-G column gave a better yield (table 1).

Table 1: Comparison of the amount of albumin isolated using two different matrices

	Concentration of Albumin (mg/*l*)	
Race Group	**CNBr – Anti-HA**	**Protein-G – Anti-HA**
Black Subjects	0.613	0.802
White Subjects	0.325	0.743

The purity of albumin isolated from crystal matrix extracts of black and white subjects was confirmed by 10 % SDS-PAGE (Fig 12) and Western blotting (Fig 13). The gel confirmed that the molecular weight of BA and WA are similar to that of commercial HSA. Since all the lanes showed a single band, it was decided that the isolated protein had been sufficiently purified and could therefore be used for crystallisation experiments.

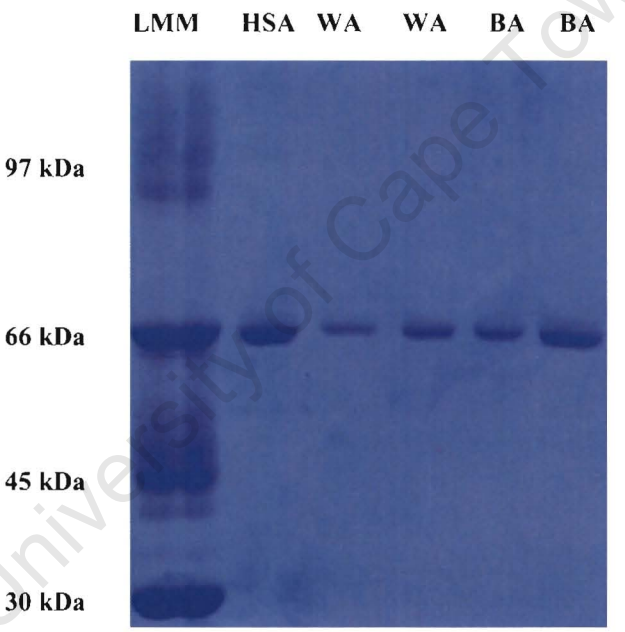


Fig 12: *10 % SDS-PAGE of albumin from black (BA) and white (WA) subjects.*

Immunodetection of the isolated protein by Western blotting (Fig 13) confirmed that the isolated protein was indeed albumin. Both high molecular weight and low molecular weight bands were observed in the commercial HSA. Wiggins et al (1985) explained that the high molecular weight bands are likely to be albumin polymers while the low molecular weight bands are albumin fragments. These authors determined that proteolysis of the albumin molecule occurred at sites both inside and outside disulphide loops (Wiggins et al, 1985). Their observation has been confirmed by Cerini et al (1999). This could explain the low molecular weight bands seen in BA and WA.

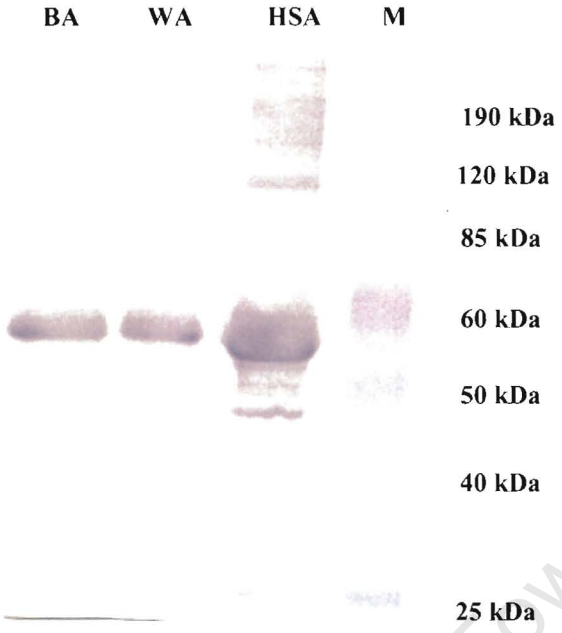


Fig 13: *Western blot of purified albumin from black (BA) and white (WA) subjects.*

2.4 Discussion

This chapter focused on the isolation of urinary albumin by immunoaffinity chromatography. Two approaches were used. The first involved the preparation of concentrated urine samples, from which albumin was isolated on a CNBr-activated sepharose column. Even with extensive dialysis and centrifugation, the concentrated samples clogged the column and reduced its efficiency. Also, the binding capacity of the column was low. 19.1 mg of anti-HA had been coupled to the CNBr matrix, yet the column was binding just under 1.00 mg as shown in Fig 7. This is because the antibody binds to the matrix via free amine groups which are present both in the Fc region and in the substrate binding regions of the antibody. Thus, the orientation of the antibody coupled to the CNBr matrix is not optimal for albumin binding. In addition, with repeated use, the column bound progressively lower amounts of albumin, possibly because anti-HA may have leached off. To avoid these problems, a new method of sample preparation was required and a different matrix was used.

One approach to establishing the association of proteins with crystals is to study them in calcium oxalate crystals freshly precipitated from healthy human urine since they will not contain proteins that are released secondarily due to tissue damage (Finlayson et al., 1961;

Khan et al., 1987; Morse et al., 1988; Morse et al., 1989; Doyle et al., 1991). Studies by Atmani et al. (2002) have demonstrated that albumin is a major constituent of the crystal matrix extract. In the present study, crystal matrix protein was extracted by demineralising crystals with EDTA solution. Analysis of the isolated protein by 10 % SDS-PAGE and Western blotting confirmed it to be albumin. The results obtained demonstrated that this was the better of the two approaches used because a higher yield was obtained.

The protein-G sepharose column was used to isolate albumin from 129 ml and 217 ml crystal matrix extract from the urine of black and white subjects respectively. 1.820 *l* of urine was obtained from 2 black subjects and 7 white subjects collected 6.610 *l* of urine. Therefore, the concentration of albumin in urine is 0.802 mg/*l* in blacks and 0.743 mg/*l* in whites. Since only two black subjects were used, the calculated concentration of albumin in blacks is not representative, nor can any conclusions be drawn whether these levels are statistically different. Cerini and co-workers have reported that on average, albumin excretion in healthy subjects is about 2.40 mg/24hr (Cerini et al., 1999).

Despite the problems encountered, albumin was successfully isolated from urine of black (BA) and white (WA) subjects and used for crystallisation experiments.

CHAPTER 3: CRYSTALLISATION EXPERIMENTS

3.1 Introduction

The inhibitory or promotory effects of urinary components or whole urine itself on CaOx crystallisation *in vitro* have been studied using different techniques (Baumann et al., 1989; Hess et al., 1996; Hess et al., 2001). This chapter considers the potential role of urinary albumin in the inhibition or promotion of CaOx crystallisation. Crystallisation experiments which were undertaken and which are described are the determination of calcium oxalate metastable limits, crystallisation kinetics, Coulter Counter measurements of particle volume-size distribution, [^{14}C]-oxalate crystal deposition rates, x-ray powder diffraction analysis, zeta-potential measurements and crystal sedimentation rates. A brief discussion of each of the techniques is presented in the following paragraphs.

Calcium Oxalate Metastable Limit

The physicochemical processes in urolithiasis have been described in chapter 1. As stated previously, urine is supersaturated with respect to CaOx; crystals form by nucleation and thereafter increase in size by crystal growth and/or aggregation (Kavanagh et al., 1993). For CaOx stone formation, it is of interest to know up to what supersaturation the metastable zone extends. One way of achieving this is by progressively increasing the oxalate ion concentration (added as NaOx) and measuring the optical density of the solution at 620nm ($OD_{620\ nm}$). The CaOx metastable limit corresponds to the lowest concentration of NaOx that causes nucleation of CaOx. This is observed as a sharp increase in $OD_{620\ nm}$ (Hess et al., 1996).

Calcium Oxalate Crystallisation Kinetics

Once the limit of metastability has been determined, supersaturation is increased in one step by the addition of oxalate at a concentration of 0.300 mM in excess of the metastable limit. The ensuing crystallisation kinetics is measured by monitoring the increase in $OD_{620\ nm}$ as a function of time. Inhibition or promotion of crystallisation in the presence of albumin (or any other inhibitor) can be estimated in this way (Kavanagh et al., 1993). The increase in $OD_{620\ nm}$ in the first few minutes is due mainly to nucleation. However, the data will always reflect the overall crystallisation process. As such, the results obtained from these experiments give a general reflection of the crystallisation potential of urine

samples. Measurements can be performed under stagnant or agitated conditions. In the latter case, extra contributions of crystal aggregation and secondary nucleation can take place and the results are system-dependent (Hess et al., 1996). In addition to CaOx crystallisation, *particle* formation may occur i.e. proteinaceous material may come out of solution and may agglomerate with crystals. Interpretation of data must take this possibility into account.

Particle Volume – Size Distribution

Another way of following crystallisation kinetics is to determine changes in particle volume-size distributions (Ryall et al., 1985; Ryall et al., 1991; Hess et al., 1996). The appearance of particles above 2.00 μm is monitored within a fixed time period by the Coulter Counter. The crystal growth rate can be determined from the slope of the linear portion of increase of particle volume with time, or from total particle volume after 90 minutes. The mean particle sizes can also be determined from the distribution (Ryall et al., 1986). Since the Coulter Counter measures particles whose sizes fall within a specified range (2.00 – 25.4 μm), crystals whose sizes lie outside this range will not be counted (Hess et al., 1996). Additionally, loose crystals containing empty spaces are recorded as if they are solid, thereby giving erroneously high estimates of crystal volume deposition. This problem is compounded by the occlusion of macromolecules into crystals. The Coulter Counter cannot account for differences in particle density. Therefore, to determine the true extent of mineral deposition, crystallisation must be induced using radioactive [^{14}C]-oxalate; the disappearance of the latter from the test solution is indicative of *crystal* formation rather than *particle* formation.

Crystal Deposition Kinetics Using Labelled [^{14}C]-Oxalate

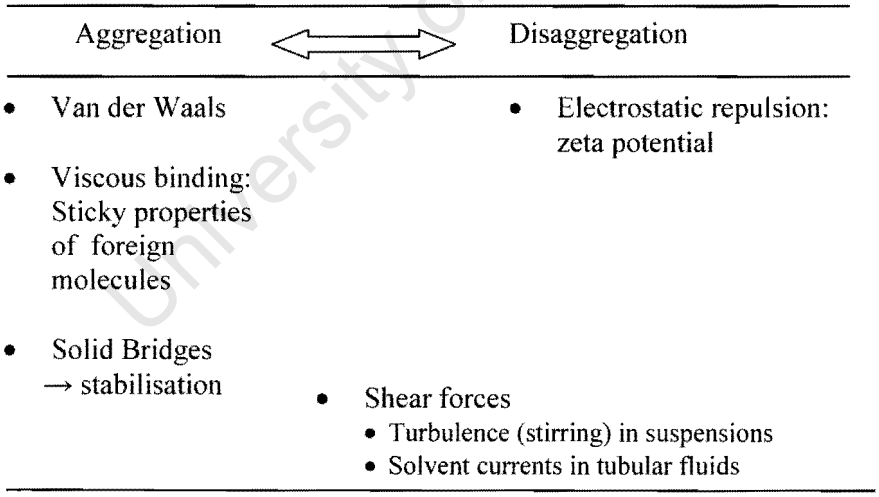
Radioactive isotopes can be used to determine the rate of precipitation of calcium oxalate from human urine in the presence and absence of inhibitors (Gill et al., 1974; Grover et al., 1998; Aggarwal et al., 2000). Because the Coulter Counter measures particles and not crystals, precipitated proteins could interfere with the data (Edyvane et al., 1987). It is therefore necessary to determine the actual amount of calcium oxalate crystals formed using labelled [^{14}C]-oxalate. The decrease in [^{14}C]-oxalate concentration at different time intervals is taken as an index of the rate of calcium oxalate crystal growth in metastable urine (Gill et al., 1974; Aggarwal et al., 2000).

X-Ray Powder Diffraction Analysis (XRD)

Irradiating a crystalline substance with x-rays produces diffracted rays. The intensity and direction of the diffracted rays depend on the unique arrangement of atoms in the crystalline sample. The intensities and spacings of the lines are then compared with a set of standards. X-ray diffraction analysis is therefore a useful technique for determining the composition and thus the purity of a crystalline sample (Sutor et al., 1968). In the present study, x-ray diffraction analysis was used to confirm the purity of calcium oxalate monohydrate (COM) crystals which were prepared for zeta potential and sedimentation experiments and also to confirm the composition of calcium oxalate crystals induced in urine samples as part of the protocol to isolate albumin.

Intercrystalline Forces and Zeta Potentials

Hess et al. (1996) have presented a summary of the forces that are presumed to be the most important for calcium oxalate crystal aggregation (Fig 14).



Intercrystalline Forces

Fig 14: *Various forces favouring aggregation or disaggregation of calcium oxalate crystals in aqueous solutions and in urine (Hess et al., 1996). Details are provided in text.*

Attractive Van der Waals forces increase strongly when interparticle distances are small, thus favouring aggregation. Aggregation is also encouraged by viscous binding of foreign compounds, such as macromolecules, to crystal surfaces. The strength of viscous binding depends on the attached foreign molecule; it might be so strong that it overcomes the

disaggregating zeta potential. Once an aggregate has been formed, it may be stabilised by solid bridges – crystalline material connecting two particles. A dual aggregating as well as disaggregating force is the shear force exerted by stirring in crystalline suspensions or by solvent currents in tubular fluids. Shear force can promote aggregation by increasing the chance of particle collision; but it can favour disaggregation by disrupting particles (Hess et al., 1996).

The charge on colloidal particles can arise from a number of different mechanisms, including dissociation of acidic or basic groups on the particle surface, or adsorption of a charged species from solution (Finlayson et al., 1984). The particle charge is balanced by an equal but opposite charge carried by ions in the surrounding liquid. These counter ions tend to cluster around the particles in diffuse clouds. This arrangement of particle surface charge surrounded by a diffuse cloud of counter charge is called the electrical double layer (Fig 15). The electrical potential drops off exponentially with distance from the particle and reaches a uniform value in the solvent outside the diffuse double layer.

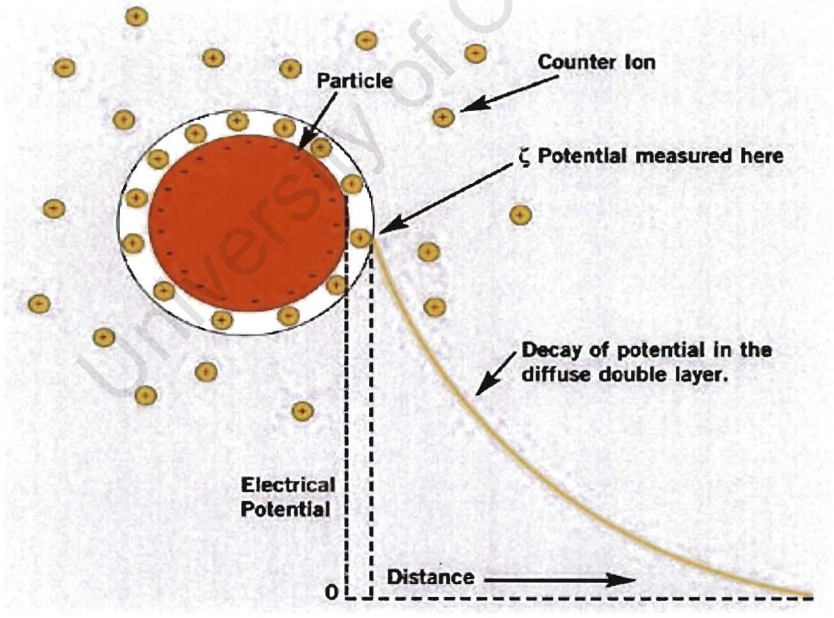


Fig 15: *A schematic diagram of the electrical double layer (http://www.bic.com/ztheory1/htm).*

The zeta potential is the voltage difference between a plane a short distance from the particle surface and the solvent beyond the double later (Finlayson et al., 1984). Thus, the zeta potential is the electrical potential at the interface between a colloidal particle (solid phase) and its surrounding solution (continuous phase) (Boeve et al., 1994). In the context

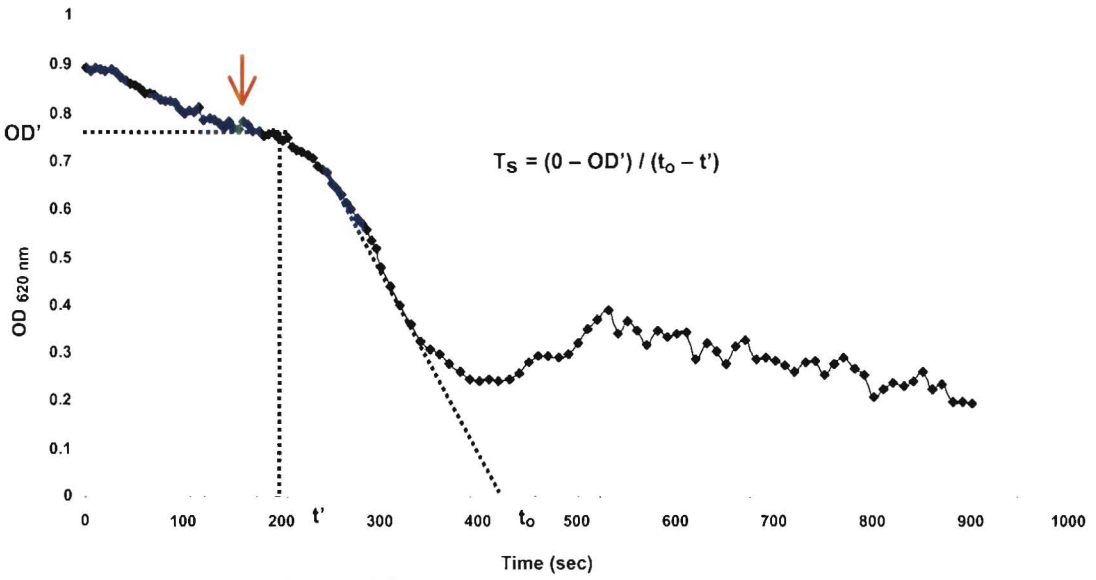
of kidney stone research, the zeta potential has been employed to study the stability of urinary colloidal systems and the mechanism of crystal aggregation. Zeta potentials have been measured for several inhibitors (Hess et al., 1989; Cao et al., 1992; Boeve et al., 1994; Cao et al., 1996). In such studies, an increase in the negative charge of the zeta potential has been interpreted as an increase in inhibition of aggregation (Scurr et al., 1986).

Measurement of Aggregation by Crystal Sedimentation

It is possible to measure COM crystal aggregation in the absence of COM crystal growth, based on the terminal sedimentation velocity of COM crystal aggregates produced by slow stirring of an equilibrated COM crystal slurry (Hess et al., 1989).

Fig 16 shows a typical time course of $OD_{620\ nm}$ of the COM crystal slurry. During slow stirring, left of arrow, $OD_{620\ nm}$ (y-axis) falls hyperbolically, to reach a plateau by 180 s. The fall in $OD_{620\ nm}$ during the 180 s is due only to aggregation, since the equilibrium solution permits neither crystal growth nor dissolution. After stirring is stopped (arrow), $OD_{620\ nm}$ falls at a rate that becomes constant at time t' (x-axis), when $OD_{620\ nm}$ is OD'. The decrease in $OD_{620\ nm}$ is due to particle settling, which is controlled by particle size. The x-intercept of the linear portion gives t_o, time by which $OD_{620\ nm}$ would have reached zero, if particles settled at constant maximal rate. The slope of the linear portion of the curve, called the turbidity slope, or T_s is $(0 - OD')/(t_o - t')$ (Hess et al., 1989). In every sedimentation experiment, the slope (T_s) of the linear regression of $OD_{620\ nm}$ versus time was calculated.

Percent of crystal aggregation in the presence of inhibitors is calculated as (T_s/T_{sc}) X 100, where T_{sc} is the turbidity slope of the control; inhibition data are presented as 100 % - %Aggregation (Hess et al., 1989).



Time Course of OD $_{620nm}$ COM

Fig 16: *Spectrophotometric determination of COM crystal aggregation in the absence of COM crystal growth modified from Hess et al., 1989. For details, see text.*

3.2 Methods

3.2.1 Subjects and Urine Collections

Single 24-hour urine samples were collected in 2.00 *l* plastic bottles from each of three male subjects in each race group. The subjects were aged 20-27 years and were recruited from students at the University of Cape Town. Individual samples were tested for blood and infection using urinalysis test strips (Combur 10 test strips; Boehringer Mannheim), then filtered with a pre-filter (Macherey-Nagel), followed by a 0.420 μm Millipore filter (Millipore Corporation, Bedford) in order to remove cellular debris. This does not affect the concentration of calcium and oxalate as inferred from previous studies (Robertson et al., 1972; Baumann et al., 2000). Urine samples were ultra-filtered using an Amicon hollow fibre bundle (H1P 10) with a nominal cut-off at 10 kDa relative molecular mass. This yielded ultrafiltered urine (UF) from black and white subjects. Prior to each crystallisation experiment, the metastable limit of the ultra filtered urine was determined.

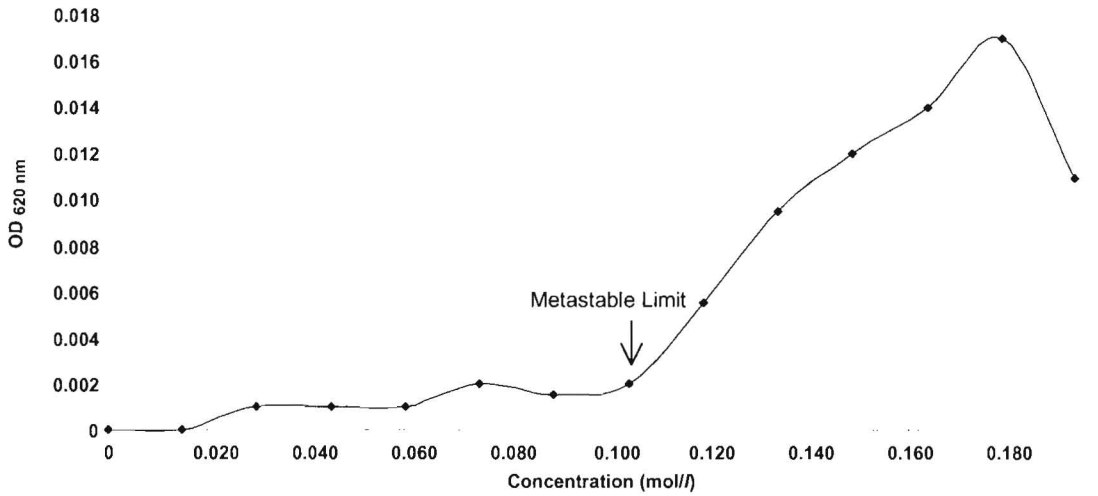
3.2.2 Metastable Limit (MSL)

0.200 M sodium oxalate (NaOx) stock solution was prepared by dissolving 2.68 g of the salt in 100 ml of water. From this stock solution, the following sodium oxalate standard solutions were prepared for metastable limit determination.

Table 1: Preparation of NaOx standard solutions

Vial	Conc. (mol/dm^3)	Stock Solution (ml)	Distilled Water (ml)
1	0	0	10.0
2	0.015	0.75	9.25
3	0.030	1.50	8.50
4	0.045	2.25	7.75
5	0.060	3.00	7.00
6	0.075	3.75	6.25
7	0.090	4.50	5.50
8	0.100	5.00	4.75
9	0.130	6.50	3.25
10	0.150	7.50	2.50
11	0.165	8.25	1.75
12	0.180	9.00	1.00
13	0.195	9.75	0.25

The metastable limit of each UF sample was determined as follows: 2.00ml UF was dispensed into each of fourteen 2ml ependorphs and incubated in an oven thermostated at 37.0 °C for 10.0 min. Tube 1, the blank, contained no NaOx. Tubes 2-14 were dosed with 20.0 μl of progressively increasing concentrations of NaOx (as shown in above table) at 2 min intervals. $OD_{620\ nm}$ (turbidity) was recorded using a spectrophotometer (Heλios Gamma and Delta, Cambridge) 30 min after each dose (n = 2). The metastable limit was determined from a plot of $OD_{620\ nm}$ vs. time, which showed the concentration of NaOx that led to spontaneous nucleation of calcium oxalate crystals – a sharp increase in $OD_{620\ nm}$ is observed (Fig 17).

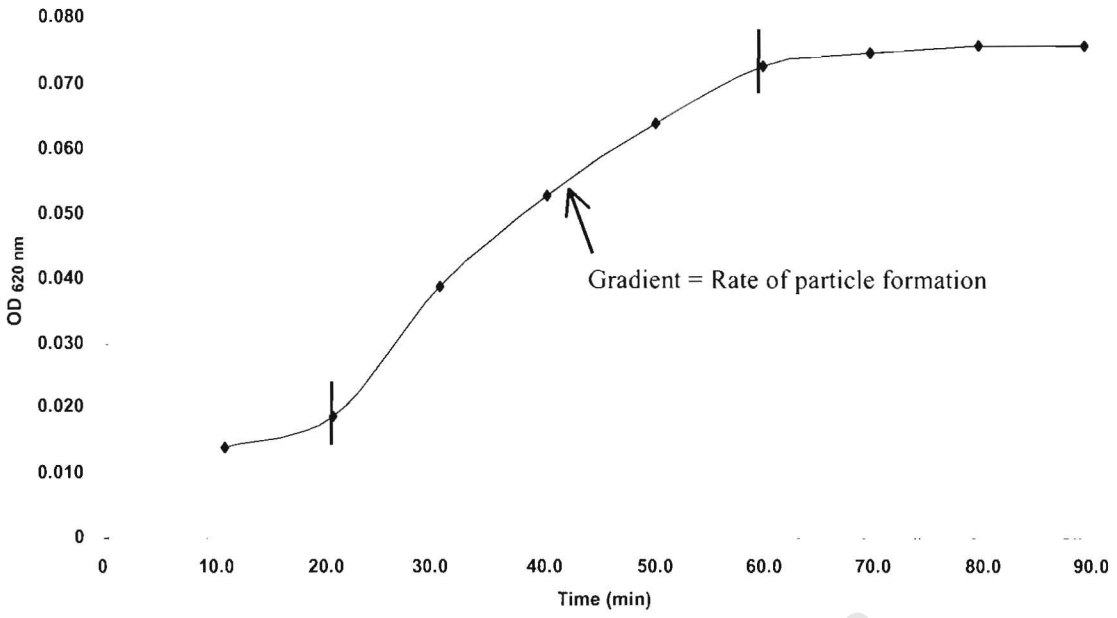


Model Graph of Metastable Limit

Fig 17: *A typical graph showing the metastable limit as a sharp rise in* $OD_{620\ nm}$.

3.2.3 Particle Formation Kinetics

One 24-hour urine sample was collected from each of three subjects in each race group. After testing for blood and infection, the samples were filtered and ultrafiltered as described previously. For each ultrafiltrate (UF), the metastable limit was determined using the above protocol, and then particle formation kinetics were determined as follows: 2.00 ml UF was dispensed into four 2.00 ml ependorph tubes. With the exception of the control, 2.00 μg of each of the commercial human serum albumin (HSA), the albumin isolated from urine of black subjects (BA) and the albumin isolated from urine of white subjects (WA) were added to the remaining three tubes respectively. The tubes were incubated in an oven thermostated at 37.0 ^{o}C for 10 min, and then dosed with 20.0 μl of NaOx at a concentration of 0.030 mol/dm^3 in excess of the metastable limit. This was taken as time t = 0. Thereafter $OD_{620\ nm}$ was measured at 10 min intervals for 90 min (n = 3). The rate of particle formation (kinetics) was determined from a graph of $OD_{620\ nm}$ vs. time. The experiment was carried out in triplicate for each individual sample. Fig 18 is a representation of a typical kinetics graph.



Typical Crystallisation Kinetics Graph

Fig 18: *Graph of $OD_{620\ nm}$ vs. time. The rate of particle formation can be determined by calculating the gradient of the linear portion of the graph.*

3.2.4 Particle Volume – Size Distribution

A 24-hour urine sample was collected from each of two subjects in each race group. After testing for blood and infection, the samples were filtered and ultrafiltered as described previously. 10.0 ml UF urine containing 1.00 μg/ml each of the HSA, the WA and the BA, as well as a control, from two individuals per race group were incubated in an oven thermostated at 37.0 °C for 10 min. After dosing with 100 μl NaOx at 0.030 mol/dm^3 in excess of the previously determined metastable limit, Coulter Counter measurements for particle volume-size and particle number-size distributions were taken at t = 90 min.

3.2.5 Measurement of [^{14}C]-oxalate Crystal Deposition Kinetics

A 24-hour urine sample was collected from each of three subjects in each race group. After testing for blood and infection, the samples were pooled and then filtered and ultrafiltered as described previously. 15.0 ml of BUF and WUF was added to four 100 ml glass flasks labelled CTRL, HSA, WA, and BA which contained 15.0 μg (1.00 μg/ml) of the respective protein. The flasks were incubated in a shaking water bath set at 37.0 °C for 10 – 15 min. 7.50 μl of ^{14}C was added, followed by 150 μl of NaOx at a concentration of 0.030 mol/dm^3 in excess of the metastable limit. This was taken as time t = 0. 2.50 ml of ultrafiltrate was taken from each flask and passed through a filter attached to a syringe into 0.250 ml concentrated HCl to quench the reaction. 1.00 ml of this mixture was transferred

into 10.0 ml scintillation fluid (n = 2). The process was repeated at 30, 60, 90, 120 min time intervals. A scintillation counter (Malvern instruments; UK) was used to monitor the amount of [^{14}C]-oxalate remaining in solution at the various time intervals. Subtraction of this value from that recorded at time t = 0 gave the quantity of precipitated CaOx. Percent precipitated oxalate vs. time was plotted to determine the effect of the different proteins on calcium oxalate crystal deposition. Since the scintillation counter measures the radioactivity due to un-precipitated [^{14}C]-oxalate, there is no significant error caused by the sticking of [^{14}C]-oxalate to the wall of the vials and the crystal-mass on the filter.

3.2.6 Preparation of Calcium Oxalate crystals

Preparation of Pure Calcium Oxalate Monohydrate Crystals for Zeta Potential Measurements and Sedimentation Experiments

COM crystals were prepared as described by Pak et al. (1975). Using two line feeds, 500 ml of 10.0 mM $CaCl_2$ and 500 ml of 10.0 mM NaOx were mixed simultaneously at room temperature at a rate of 1.00 ml/min using a peristaltic pump. The mixture was then stirred for a week at 6.00 °C. Crystals were then filtered through a 0.220 μm filter paper, washed with 2.00 ml of methanol and dried in an oven at 95.0 °C for an hour. X-ray powder diffraction analysis was used to confirm the presence of COM.

Preparation of Calcium Oxalate Crystals in whole Urine for Protein Isolation

24-hour urine specimens were collected from 2 healthy black and 2 healthy white male subjects and were then pooled after confirming the absence of blood and infection. An adapted method of that described by Atmani et al. (2002) was used for the induction of calcium oxalate crystallisation in whole urine. Pooled urines from the race groups were warmed to 37.0 °C in a shaking water-bath. CaOx crystallisation was induced by drop-wise addition of 15.0 ml/*l* of 100 mM sodium oxalate solution at pH 5.90 - 6.50. After 1 hour, the same amount of sodium oxalate was added to the mixture. An hour later, the crystals were harvested by centrifugation at 8000 *g* using a JR-20 rotor (Beckman Instruments, Fullerton, Calif. USA) for 30 min at room temperature. The calcium oxalate crystals grown in whole urine from black and white subjects were then analysed by x-ray powder diffraction.

3.2.7 Zeta Potential Measurements

Four vials containing 11.0 ml 0.300 mg/ml COM crystal slurries were prepared in 10.0 mM sodium acetate buffer, pH 5.70 and were equilibrated overnight with constant stirring (1100 rpm) at 25.0 °C (Hess et al., 1989). Besides the control, which had no protein, 1.00 μg/ml each of the HSA, the WA and the BA was added to each of three vials respectively and stirring continued for 2 hours at 37.0 °C and 1100 rpm. The zeta potential of the COM slurry in each vial was measured in duplicate using a Zetasizer 4 (Malvern instruments; UK). Five different runs were carried out and a mean obtained for the effect of each protein on the zeta potential of the COM crystal slurry.

3.2.8 Rate of Sedimentation

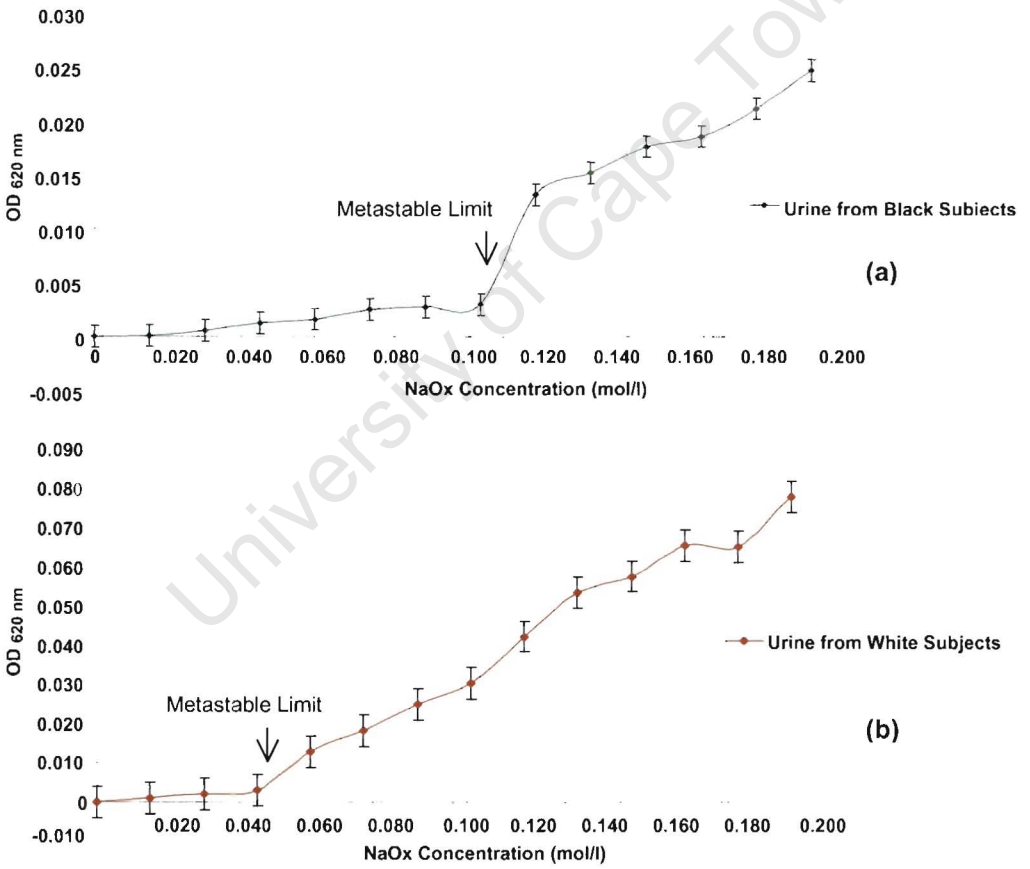
The spectrophotometric assay method developed by Hess et al. (1989) was used for the measurement of COM crystal aggregation in the absence (control), and presence of albumin (HSA, BA and WA). COM crystals prepared as described in section 3.2.6 were dissolved in 10.0 mM Tris-HCl buffer, containing 90.0 mM NaCl, pH 7.50 to yield COM slurries. The concentration of the COM crystal slurries was 0.800 mg/ml. Four vial containing 7.00 ml 0.800 mg/ml crystal slurries were equilibrated overnight in a water jacket set at 37.0 °C, with continuous stirring at 1100 rpm. After equilibration, 1.00 μg/ml each of the HSA, the WA and the BA was added to three vials respectively and stirring continued for 2 hours at 37.0 °C and 1100 rpm.

A 2.00 ml aliquot of slurry was then transferred into a 10.0 mm cuvette in a cell holder thermostated at 37.0 °C, with continuous stirring at 1100 rpm. $OD_{620\ nm}$ was measured at 5.00 sec intervals for 5 min, 10.0 sec intervals for 10 min or until $OD_{620\ nm}$ was unchanging. At this point, the stirrer was turned off, and $OD_{620\ nm}$ was recorded until there was no more change in absorbance due to particle settling (Hess et al; 1989). Three sets of $OD_{620\ nm}$ were obtained per run for each vial - the control (CTRL), HSA, BA and WA. In each sedimentation experiment, the slope (T_s) of the linear regression of OD_{620nm} versus time was calculated. Percent of crystal aggregation in the presence of protein was calculated as (T_s/T_{sc}) X 100, where T_{sc} is the turbidity slope of the control. Percentage inhibition is therefore represented as 100 % - %Aggregation (Hess et al., 1989). A total of five runs were carried out on five different days and an average obtained.

3.3 Results

3.3.1 Metastable Limit (MSL)

It is well-known that urine is supersaturated with respect to CaOx (Nordin et al., 1993; Hess and Kok; 1996; Baumann et al., 1997). Determining the metastable limit of a urine sample provides the investigator with information on the tendency for crystals to form, and is also an indication of the urine sample's ability to inhibit crystal nucleation. The metastable limit is of great practical significance for excretion of poorly soluble salts as it represents the boundary of supersaturation that might be sustained without formation of crystals or stones (Kavanagh, 2000). The mean plots of OD_{620nm} vs. concentration of NaOx for the determination of metastable limits in the urines of black and white subjects are given in Fig 19 (a) and (b) respectively.



Metalstable Limit

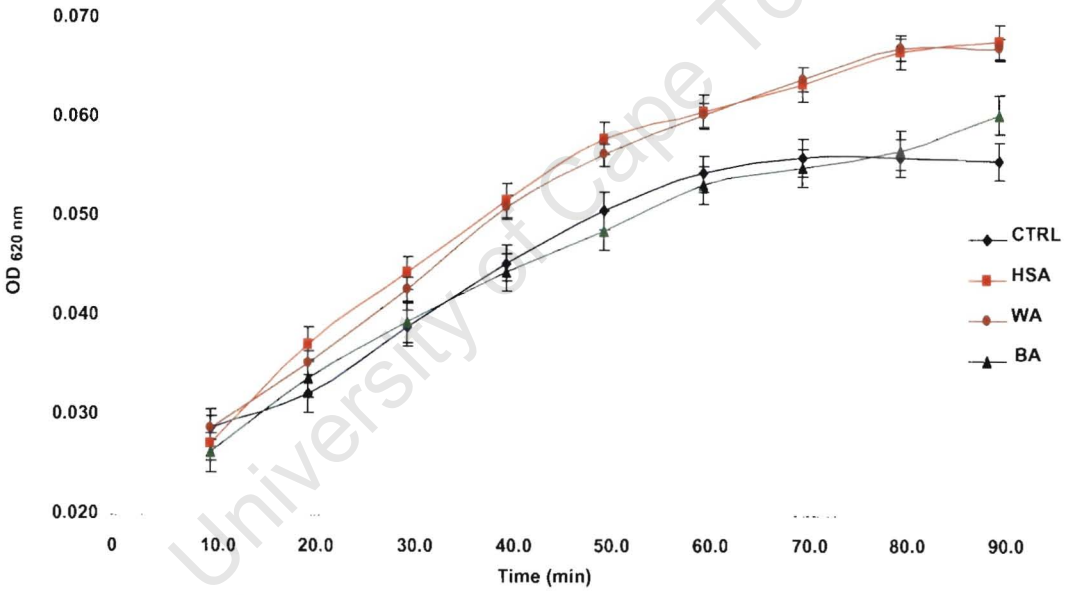
Fig 19: *Mean plots for the determination of metastable limit of urine samples from 8 black subjects (a) and from 9 white subjects (b).*

These plots show that CaOx crystal formation in the urine of blacks is more difficult to initiate than in the urine of whites. This supports the general observation that kidney stone disease occurs rarely in the black race group. Urine composition and physicochemical data for the various urines are given in appendix II, pages 106-7.

3.3.2 Particle Formation Kinetics

As mentioned previously, supersaturation is the driving force of crystallisation. In a supersaturated solution, the resultant kinetics of crystal formation consists of one or more of the following mechanisms - nucleation, crystal growth, crystal aggregation and phase transformations (Hess and Kok; 1996). The rates at which these processes occur will determine the characteristics of the inorganic material formed, such as crystal phase, particle shape, particle size and number of particles. These rates in turn are dictated by the solution chemistry (Hess and Kok; 1996).

The effect of 1.00 μg/ml each of HSA, BA and WA on calcium oxalate crystallisation kinetics in BUF, relative to the control is shown in Figs 20 and 21.

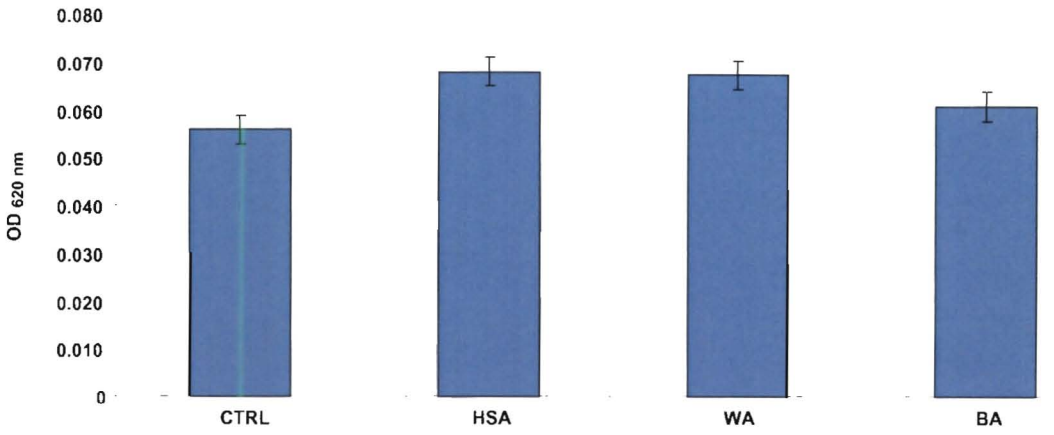


Mean Plots of OD_{620nm} Vs Time

Fig 20: *Graph of $OD_{620\ nm}$ vs. time from which the rate of particle formation in BUF in the presence and absence of albumin was determined.*

The rate of particle formation in BUF as determined from the gradients of the above curves follows the trend CTRL = BA < WA = HSA.

After 90 min incubation, the total amount of crystal formation, measured as $OD_{620\ nm}$ is shown in Fig 21.

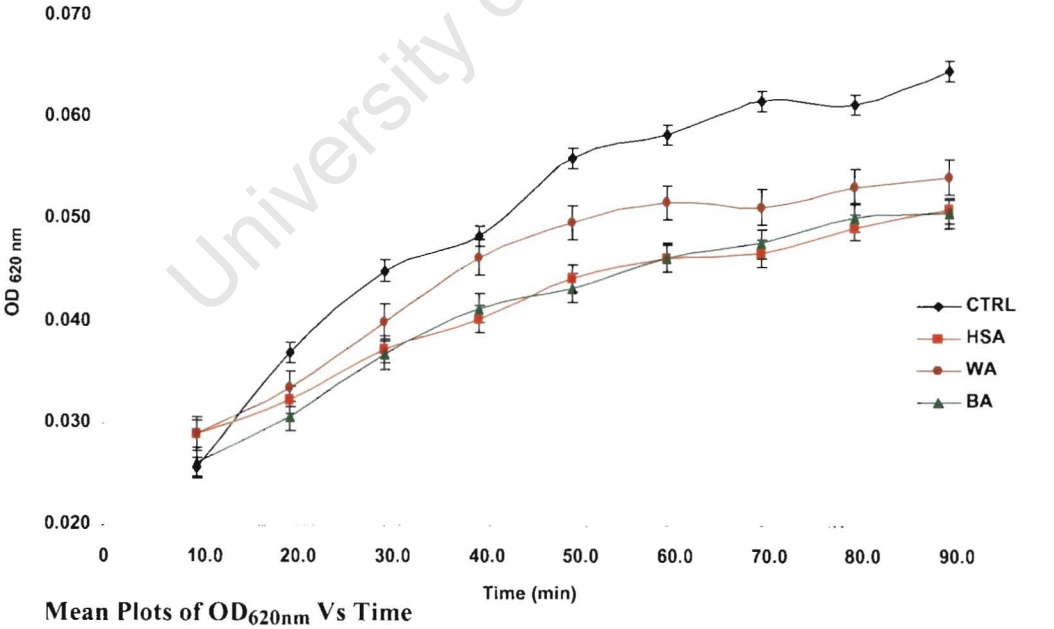


OD_{620nm} in BUF after 90min

Fig 21: *A comparison of the total amount of particles present in BUF containing the different proteins after incubation with NaOx for 90 min.*

The trend observed after 90 min is CTRL<BA<WA=HSA. These results indicate that the proteins promote CaOx crystal formation in BUF. WA and HSA have a greater effect in BUF than BA.

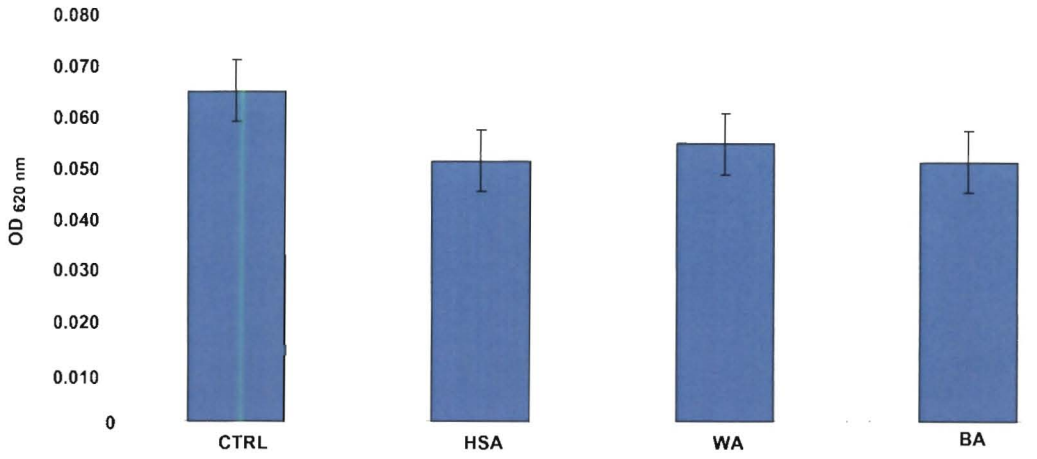
Results obtained for the effect of 1.00 μg/ml each of HSA, BA and WA on calcium oxalate crystallisation kinetics in WUF are shown in Figs 22 and 23.



Mean Plots of OD_{620nm} Vs Time

Fig 22: *Graph of $OD_{620\ nm}$ vs. time from which the rate of particle formation in WUF in the presence and absence of albumin was determined.*

The rate of particle formation in WUF as determined from the gradients of the above curves follows the trend BA = HSA < WA < CTRL.



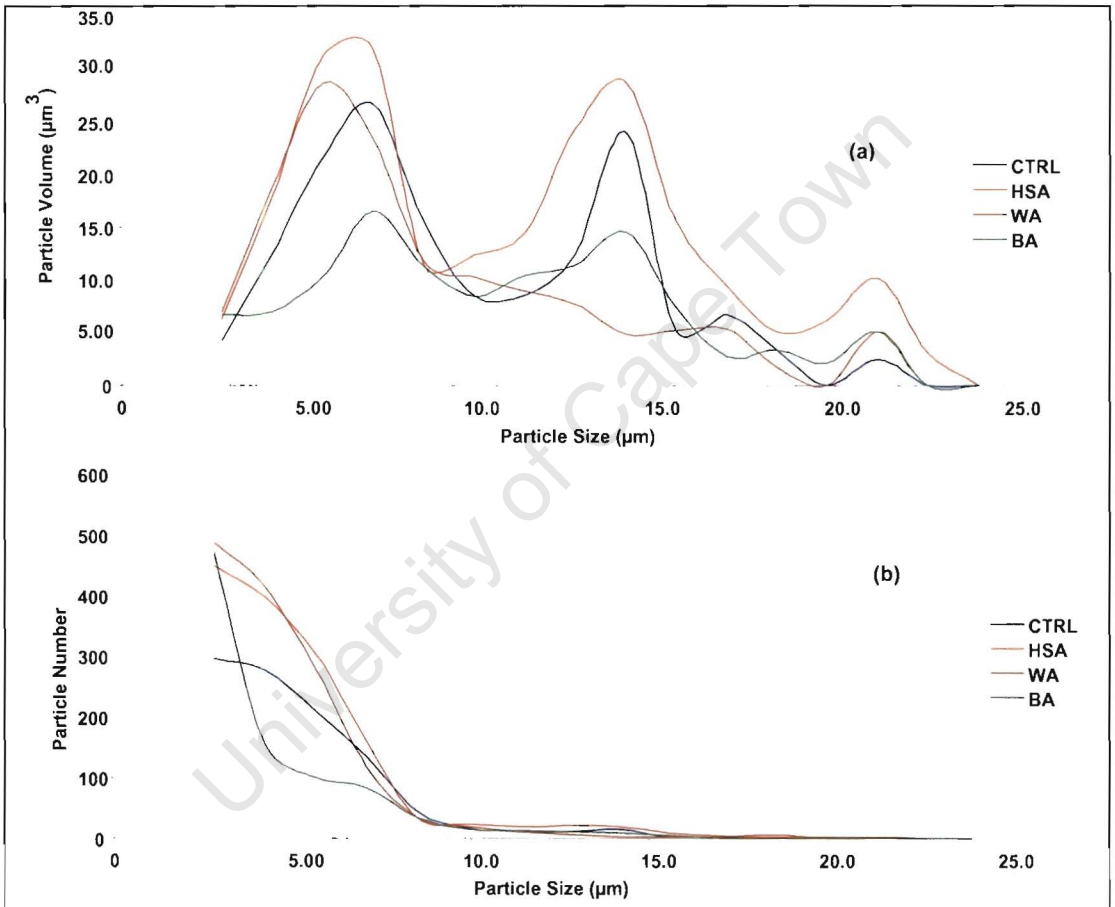
OD_{620nm} in BUF after 90min

Fig 23: *A comparison of the total amount of particles present in WUF containing the different proteins after incubation in NaOx for 90.0min*

The trend observed after 90 min is BA=HSA<WA<CTRL. These results indicate that the protein inhibits CaOx crystal formation in WUF.

3.3.3 Particle Volume -Size Distribution

The Coulter Counter was used to monitor formation of particles above 2.00 μm at time t = 90 min after dosing with NaOx solutions. The particle volume-size distribution and particle number-size distribution are given in Fig 24. In general, a decrease in total particle volume can arise from three possible events – fewer particles, smaller particles or less aggregation (Hess et al., 1995). Particle volume-size and particle number-size distributions are given in Fig 24 (a) and Fig 24 (b) respectively.

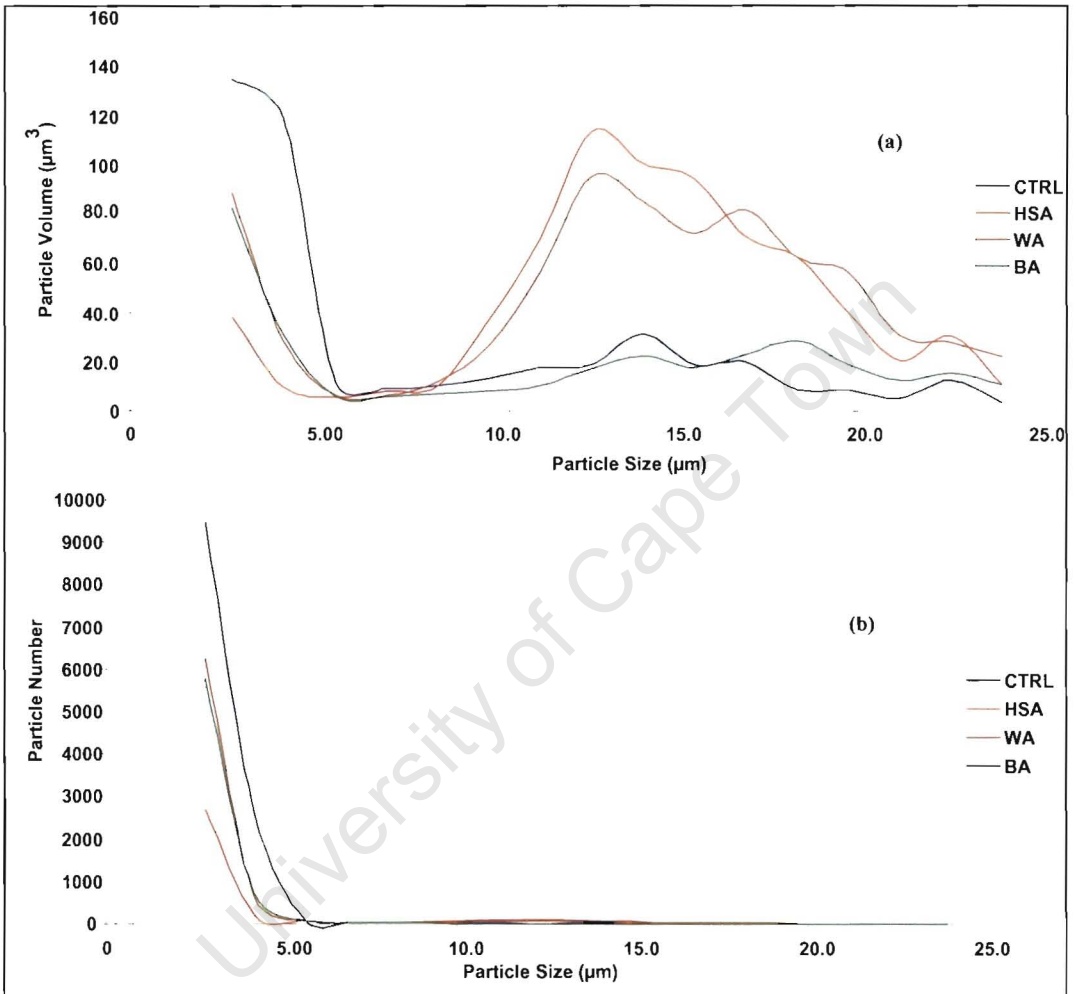


Particle Volume-Size and Particle Number-size Distributions in BUF

Fig 24: *The above distribution curves are for particle volume – size (a) and particle number – size (b) that was obtained in urine from black subjects at time t = 90.0min*

It is observed that in BUF, particle volumes and particle numbers are relatively small. The volume-size distribution, Fig 24 (a), shows 3 peaks at approximately 7 μm, 14 μm and 21 μm. However, the number-size distribution, Fig 24 (b), shows only one ill defined peak < 5 μm. Because of the low particle numbers, the volume distribution may be strongly influenced by single atypical crystals. As such, the volume-size distribution will not be considered further. The number-size distribution shows the trend, CTRL<BA=WA=HSA.

Particle volume-size and particle number-size distributions in WUF are given in Fig 25 (a) and Fig 25 (b) respectively. It is noted that particle numbers are an order of magnitude greater than in BUF. Volume-size (Fig 25a) and number-size (Fig 25b) trends tend to agree, albeit that the peaks are not well defined. The trend appears to be HSA<BA=WA<CTRL.



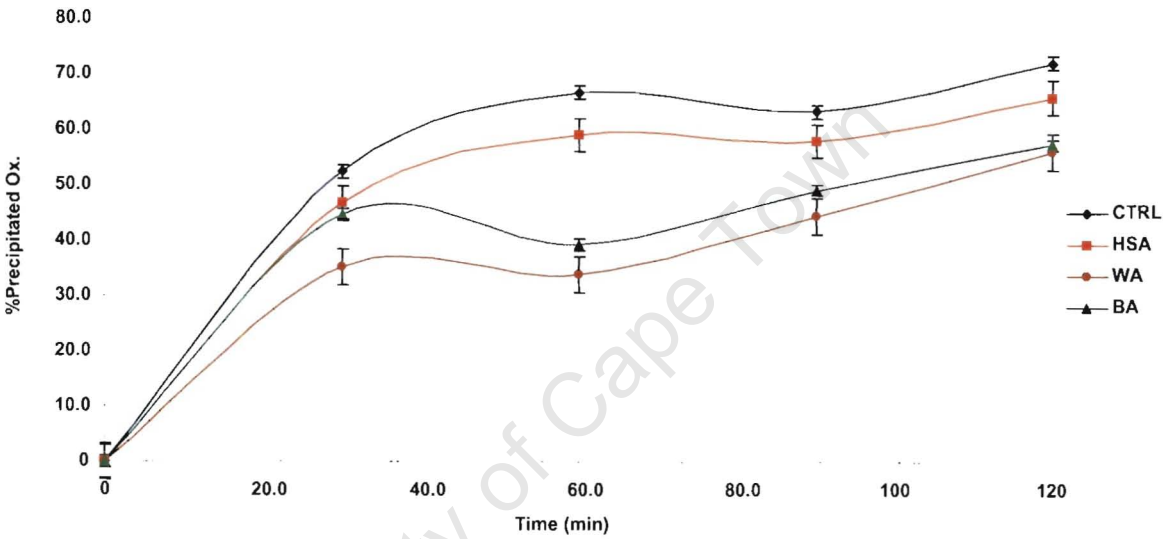
Particle Volume-Size and Particle Number-size Distributions in WUF

Fig 25: *Particle volume – size (a) and particle number – size (b) distribution curves obtained in urine from white subjects at time t = 90 min*

The additional peaks for HSA and WA (at approximately 13 μm) in the volume-size distribution and their absence in the number-size distributions again suggest the possible presence of single atypical crystals. As in BUF, these peaks will not be considered further.

3.3.4 Crystal Deposition Kinetics Using Labelled [^{14}C]-Oxalate

The effect of albumin on the deposition of labelled [^{14}C]-oxalate in BUF is shown in Fig 26. As explained earlier, the Coulter Counter has an inability to distinguish crystals from particles which may result in precipitated proteins being incorrectly interpreted as crystals. A decrease in [^{14}C]-oxalate concentration, monitored using a scintillation counter, at different time intervals provides a measure of the rate of CaOx *crystal* formation in metastable urine (Gill et al., 1974; Aggarwal et al., 2000).

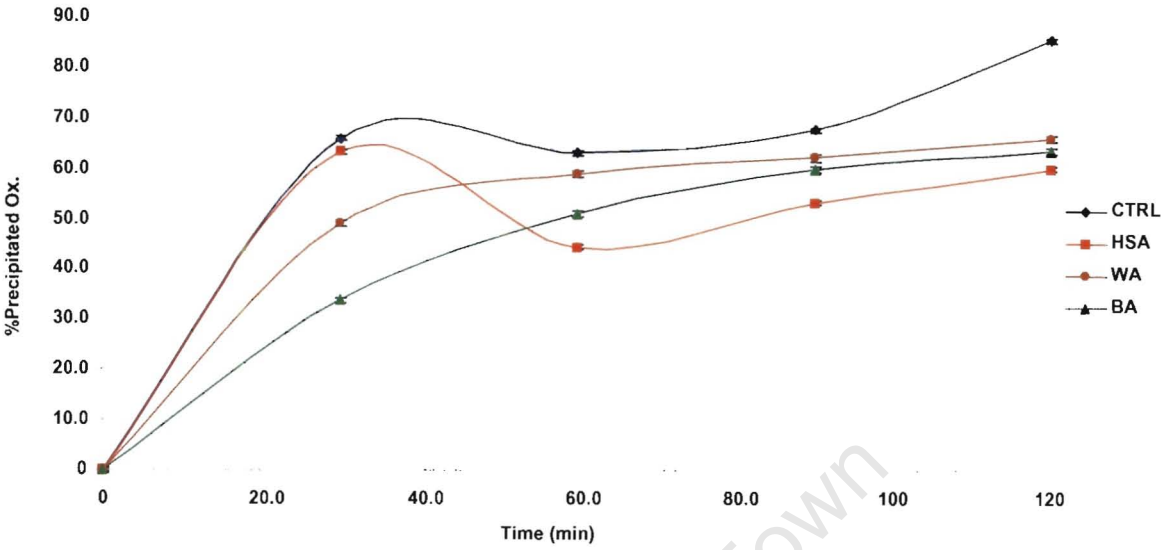


Albumin's Effect on [^{14}C]-oxalate Crystal Deposition in Pooled BUF

Fig 26: *The rate of CaOx crystal deposition in pooled BUF in the presence and absence of albumin is given by the gradient of the linear section of the curve, from t = 0 to t = 30 min*

The CaOx deposition rates in BUF as determined by the gradients of the curves during the first 30 min follows the trend WA<BA=HSA<CTRL. Thus, WA, BA and HSA inhibit CaOx deposition (Fig 26).

Albumin was found to inhibit CaOx deposition in WUF as well (Fig 27). The trend for the deposition rate was BA < WA < HSA = CTRL.



Albumin's Effect on [^{14}C]-oxalate Crystal Deposition in Pooled WUF

Fig 27: *The rate of CaOx crystal deposition in pooled WUF in the presence and absence of albumin is given by the gradient of the linear section of the curve, from t = 0 to t = 30 min*

3.3.5 X-Ray Powder Diffraction Spectra for CaOx Crystals

Fig 28 below indicates the relative intensity and interplanar spacings (Å) of calcium oxalate crystals prepared for (a) zeta potential and sedimentation measurements and for protein isolation from the urine of (b) black and (c) white subjects.

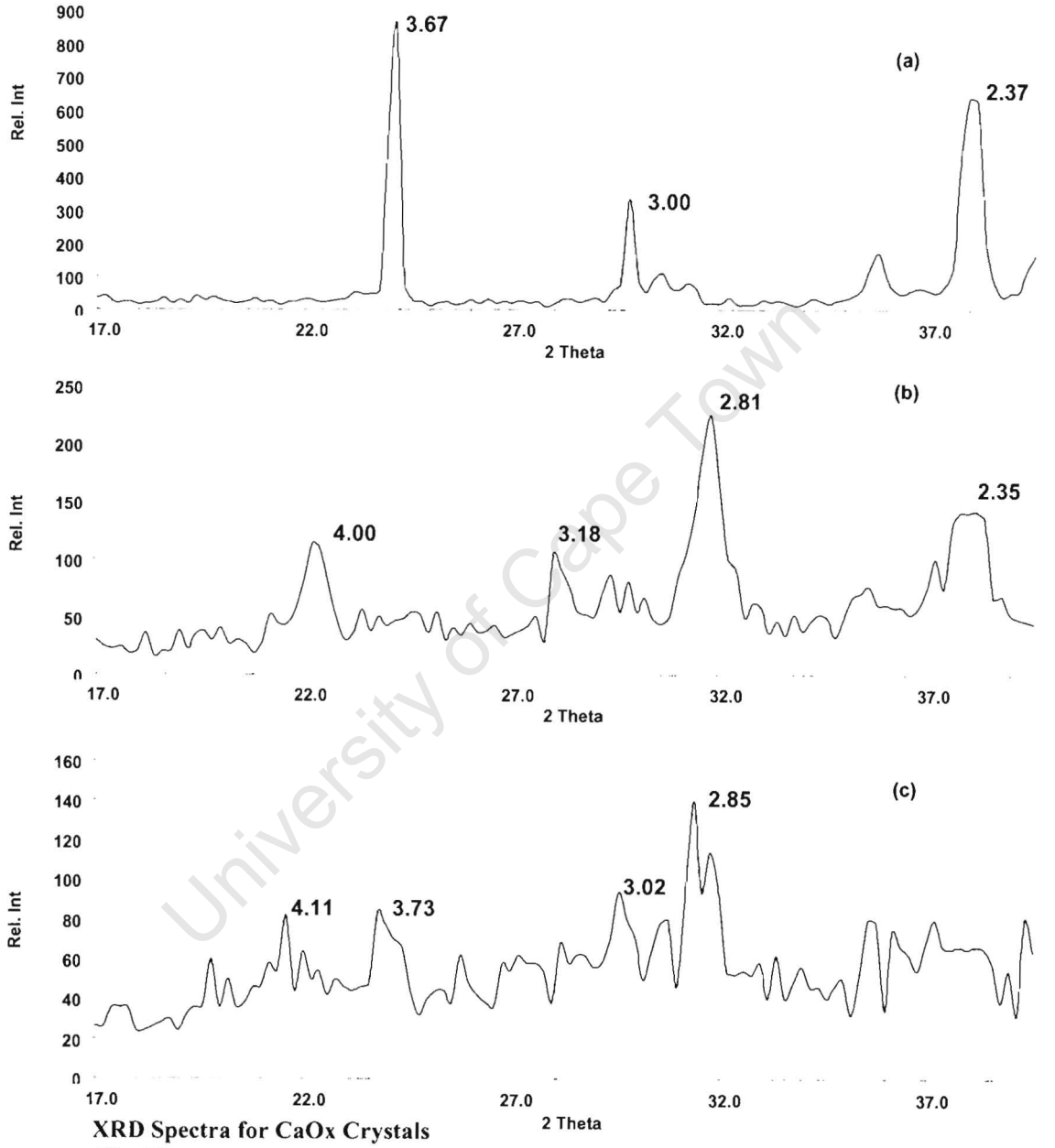


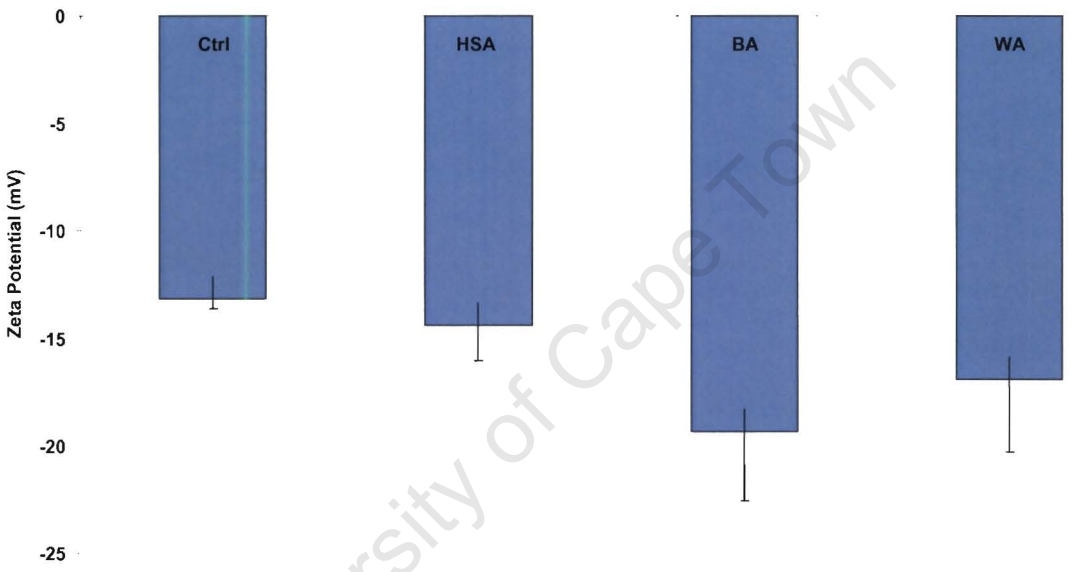
Fig 28: *X-ray Powder diffraction spectra for calcium oxalate crystals. (a) Crystals prepared for zeta potential and sedimentation measurements using the method of Pak et al. (1975). For (b) and (c), crystals were precipitated from urine of black and white subjects respectively using the method of Atmani et al. (2002).*

Comparison of the d-spacing (figures in bold) with standard tables published by Sutor et al (1968) indicates that Fig 28 (a) is pure COM. The spectrum obtained for Fig 30 (b) is characteristic of calcium oxalate dihydrate (COD). Fig 28 (c) is difficult to interpret due to noise interference. However, the peak at 2.85 Å is characteristic of COD and that at 3.02

Å is close to the COM characteristic peak of 2.97 Å. The peak at 3.73 Å is close to characteristic peaks of both COM and COD. Thus it appears that urine from black subjects produce mainly COD, while urine from white subjects produce a mixture of COM and COD when challenged with NaOx.

3.3.6 Zeta Potential Measurements

Fig 29 shows histograms for the mean zeta potentials of crystal slurries in the presence and absence of 1.00 μg/ml each of the HSA, the WA and the BA.



Effect of Urinary Albumin on Zeta Potential

Fig 29: *Mean zeta potentials of crystal slurries in the absence and presence of albumin.*

The negative charge (zeta potential) of COM crystal slurries increased in the presence of all three proteins. Values followed the sequence BA (-19.3 mV), WA (- 16.9 mV), HSA (-14.2 mV) and COM control crystals (–13.0 mV). BA carries a more negative charge than WA and will therefore exert stronger repulsive forces and is likely be a superior inhibitor of crystal aggregation.

3.3.7 Rate of Sedimentation

The spectrophotometric assay used for the measurement of aggregation by crystal sedimentation yielded the results shown in Fig 30. The crystal slurries contained 1.00 μg/ml each of the HSA, the BA and the A.

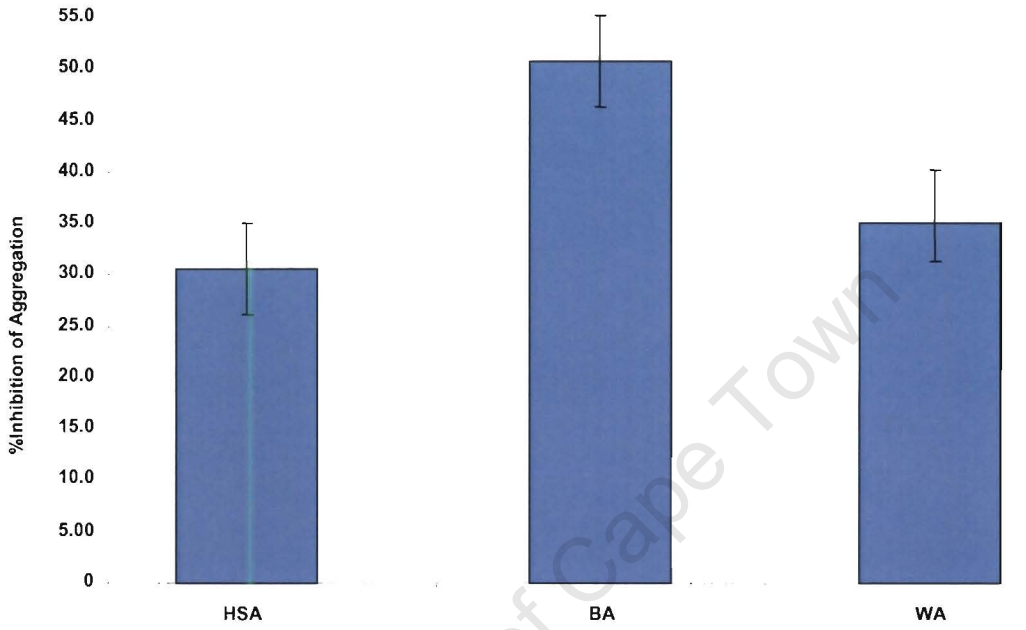


Fig 30: *The percentage inhibition of COM crystal aggregation by HSA, BA and WA.*

The percentage inhibition of COM crystal aggregation by HSA and WA was not significantly different (31.3 % and 35.1 % respectively). However, inhibition of COM crystal aggregation by BA was significantly higher (50.6 %).

3.4 Discussion

The main reason why stone disease does not occur in all persons is that normal urine retards and inhibits crystallisation (Pak et al., 1976; Coe et al., 1991). To define the role of inhibitors in the formation of crystals and stones, it is customary to investigate crystallisation *in vitro* in the presence and absence of individual macromolecules, using either aqueous inorganic solutions, or urine itself (Ryall et al., 1995; Asplin et al., 1997; Maslamani et al., 2000). In the current study, albumin from black and white subjects was investigated for its effect on CaOx crystallisation in inorganic media as well as in ultrafiltered urine from both race groups.

Previous work on albumin has shown that it has no effect on the metastable limit (Edyvane et al., 1987). As such, this study did not investigate the effect of albumin on this urinary parameter but simply compared the difference in the metastable limits of ultrafiltered urine samples obtained from black (BUF) and white (WUF) subjects without the addition of albumin. Fig 19 shows that the limit of metastability was 0.100 mol/dm^3 and 0.045 mol/dm^3 for BUF and WUF respectively. These results together with the lower RS_{CaOx} values in blacks compared to whites (appendix II, pages 106-7) suggest that urine from black subjects can tolerate higher levels of supersaturation without the formation of CaOx crystals, compared to urine from white subjects and as such, is in agreement with the significantly lower incidence of urolithiasis in this race group. The obvious question which arises is "does urine from black subjects contain inhibitors of CaOx crystallisation which are more potent than those which occur in urine of white subjects?"

Before drawing conclusions regarding the inhibitory or promotory effects of albumin, it is essential that the data acquired by spectrophotometry, Coulter Counter and radioactive [^{14}C]-oxalate be clearly understood and interpreted.

Both spectrophotometry and Coulter Counter techniques suffer from the same limitation regarding their inability to distinguish between a "particle" and a "crystal" of calcium oxalate. Thus, an increase in optical density (in the case of spectrophotometry) or an increase in the number of particles (in the case of Coulter Counter) cannot be confidently attributed to the formation of only CaOx crystals. Other particulate matter (such as proteins) could be co-precipitating independently of the crystals or could be adsorbed onto

the crystal surfaces. The only way to measure CaOx crystal formation *per se* is to use a [^{14}C]-oxalate deposition assay in which the rate of disappearance of the latter from the test solution provides a direct and quantitative measure of CaOx crystallisation kinetics. Thus, the data acquired by the three techniques – spectrophotometry, Coulter Counter, [^{14}C]-oxalate deposition – must be considered in concert.

In BUF, the [^{14}C]-oxalate deposition rates followed the trend WA<BA<control, indicating that WA is a stronger inhibitor than BA. However, the results for spectrophotometry and for Coulter Counter (particle number) both show the trend to be CTRL $\leq$ BA $\leq$ WA, thereby indicating a significant amount of independent protein co-precipitation. (Protein adsorption onto CaOx crystal surfaces is probably also occurring but cannot be positively identified by these of data).

In WUF, the formation rates for all the techniques fit a general trend BA $\leq$ WA $\leq$ CTRL. Thus, it can be concluded that in WUF, albumin from both race groups inhibits the rate of formation of CaOx crystals and that BA is more efficacious in this regard. In addition, it appears that co-precipitation of protein, if it occurs at all, is not an independent process i.e. it is probably being adsorbed directly onto CaOx crystal surfaces and as such, does not significantly increase the optical density or the number of particles.

It is interesting to consider the above results in the light of previous studies. Reports of the effect of albumin on crystal growth in literature are contradictory. In some studies, its effect has been described as modest or absent depending on experimental conditions (Edyvane et al., 1987; Worcester et al., 1988; Grover et al., 1998; Chen et al., 2001).

In a dilute seeded crystallisation system, albumin was found to be a weak inhibitor of crystal growth (Edyvane et al., 1987; Chen et al., 2001). On the other hand, Worcester et al. (1988) reported that albumin has a strong affinity for COM crystals, yet did not inhibit crystal growth even at concentrations as high as 3×10^{-6} M. They therefore proposed that either almost all of the adsorption occurred at non-growth sites or the protein adsorbed to growth sites but did not prevent growth. Similar results were obtained by Grover and associates (1998) who found that crystal growth, as measured by [^{14}C]-oxalate disappearance was unaffected by albumin. In all of the above studies (Edyvane et al., 1987; Worcester et al., 1988; Grover et al., 1998; Chen et al., 2001) commercial HSA and

not urinary albumin (as in the present study) was used. Also, the assay system used in the present study was different to that used by Edyvane et al. (1987), Worcester et al. (1988) and Chen et al. (2001) but similar to that used by Grover et al. (1998). However, in the latter study, the researchers carried out their investigations in inorganic medium and not in real urine as was the case in the present study. Thus, the fundamental difference between the protocols in previous studies and the present one could account for the different results.

The current study has a significant advantage over the above mentioned ones because urinary albumin was used in real urine in order to determine its role in the inhibition of crystal growth. This is preferable to inorganic solutions because it more closely represents the environment in which stones are formed. It is clear from Figs 26 and 27 that albumin from both race groups inhibits CaOx crystal deposition. Also, their roles in urine from the different race groups are reversed. BA is a more potent inhibitor of CaOx crystal growth in WUF, while in BUF, WA displays a greater inhibitory effect than BA. Therefore, the nature of urine composition appears to affect albumin's performance. Perhaps the nature of urine influences the degree of aggregation of albumin.

Other studies have revealed that in the presence of albumin, crystals which formed were smaller and more numerous than those in control solutions (Edyvane et al., 1987; Ryall et al., 1991; Hess et al., 1995; Grover et al., 1998; Cerini et al., 1999). This apparent promotory effect of albumin on CaOx precipitation has been ascribed to the protein's ability to act as a microsubstrate for the deposition of calcium and oxalate ions and thereby induce heterogeneous nucleation of CaOx crystals (Ryall et al., 1987). Initiation of crystal nucleation would require that the protein provide an adequate template for the positioning of the ions that would form the first crystal lattice. Cerini et al. (1999) proposed that the Ca^{2+} ions would bind preferentially to carbonyl or carboxyl groups on the protein. Then, because CaOx crystals present faces composed of alternate layers of calcium and oxalate ions, by diffusion and adsorption of calcium over the protein surface, albumin could stabilize calcium interactions by ionotropic effect and initiate crystal nucleation by ion pairing, allowing binding to oxalate ions (Cerini et al., 1999). The formation of smaller crystals in greater numbers, as described above, was not observed in the present study, possibly because real urine was used in the latter. Albumin, irrespective of its origin, has been shown in the present study to be an inhibitor of CaOx crystallisation. However, the origin of the urine in which the albumin was tested, had an effect on the protein's

behaviour. In BUF, proteins from both race groups formed fewer crystals and their total volume was less than those crystals which formed in WUF. This observation demonstrates a synergistic relationship between protein inhibitory performance and urine environment. Similar results have been obtained for urinary proteins such as UPTF1 (Durrbaum et al., 2001; Webber et al., 2003) and THM (Craig et al., 2001).

Crystal aggregation is considered to be more important for stone formation than crystal nucleation and growth (Tiselius et al., 1995; Hess et al., 1996). The reason for this is that calcium oxalate growth is too slow to produce clinically significant particles within the transit time of urine in the urinary tract, whereas aggregation occurs within seconds and is thus more dangerous for the formation of large crystalline particles in renal tubules (Tiselius et al., 1995; Hess et al., 1996). Inhibition of crystal aggregation is related to changes in surface charge that occur when a macromolecule, like albumin, binds to crystals. The surface charge or zeta potential becomes more negative. This increases the electrostatic repulsive forces between crystals.

In the present study, the negative charge (zeta potential) of the COM crystal slurries increased in the presence of all three proteins relative to the control i.e. the charge became more negative (Fig 31). Values followed the sequence BA (-19.3 mV) > WA (-16.9 mV) > HSA (-14.2 mV) > COM control crystals (–13.0 mV). These zeta potential values demonstrate that in the presence of BA, COM crystals carry a more negative charge than in the presence of WA. As such, BA will exert stronger repulsive forces and is likely to be a superior inhibitor of crystal aggregation.

The effect of albumin on CaOx aggregation was also investigated by measuring the terminal sedimentation velocity of COM crystal aggregates produced by slow stirring of an equilibrated COM crystal suspension. The results (Fig 30) show that albumin (irrespective of its origin) is an inhibitor of COM crystal aggregation and that BA is superior to WA and HSA in this regard. Albumin's inhibition of aggregation agrees with literature where it has been reported to inhibit COM crystal aggregation detectably at a concentration of 0.01 μM (Hess et al., 1989). This trend confirms the interpretation of the zeta potential data which suggested that BA is a more potent inhibitor of COM crystal aggregation than HSA and WA. Since aggregation is probably the most crucial mechanism in stone formation, BA's

ability to inhibit this process may be one of the factors responsible for the rare occurrence of urolithiasis in black South Africans.

In the conditions under which the zeta potential measurements were performed (acetate buffer, pH 5.7), some free calcium would be bound to the acetate and the free calcium / free oxalate ratio will be <1. In the sedimentation rate experiments, a Tris-HCl buffer, pH 7.5 was used. Here more free calcium ions would be available, the free calcium / free oxalate ratio will approach unity and possibly, albumin is more protonated. In order to account for the pH difference in the two procedures, further studies will aim at investigating the behaviour of BA, WA and HAS under different calcium concentrations and pH values.

X-ray diffraction data obtained in the present study demonstrated that urine from healthy black and healthy white subjects contained COD crystals and a mixture of COM and COD crystals respectively. This was contrary to expectations since recent literature has reported that urine from black subjects produced predominantly COM when NaOx is added (Webber et al., 2003). The observed results cannot be attributed solely to albumin since the crystals were induced in whole urine. With pure compounds, it has been found by several investigators (Furedi-Milhofer et al., 1995; Wesson et al., 1998; Sikiric et al., 1999; Wesson et al., 2000) that an inhibitor can stimulate COD formation by completely blocking COM nucleation. The driving force of supersaturation has to go somewhere, COM is preferred but when the route to COM is blocked COD will form. In future studies, scanning electron microscopy can be used to support the x-ray diffraction data as well as to show whether specific crystal surfaces of COM are being affected by adsorption of albumin to the crystals. It will also be worthwhile to perform nucleation experiments in the presence of increasing concentrations of albumin. This will provide information on whether albumin stimulates or inhibits nucleation and in addition show if albumin has a preference for a specific crystal surface. Furthermore, it may show differences in action between BA, WA and HSA. A molecular basis for the observed inhibitory differences will also be explored by conducting biochemical analysis of the different proteins.

The formation of COD over COM in the urine of healthy black subjects would be advantageous because the interaction of COM crystals with renal cells has been shown to result in cell death (Koul et al., 2002). Additionally, the positive charge on COD crystals

is higher than that on COM crystals (Wesson et al., 1995). The higher positive charge on COD crystals would prevent their adhesion to the cell surface and therefore inhibit their retention in the renal tubes (Wesson et al., 1995; Cerini et al., 1999). Thus, the production of COD in urine of black subjects affords them protection from stone disease because the COD crystals are less likely to bind to the renal epithelial cells and be retained in the kidney.

CHAPTER 4: CONCLUSION

A study of the effect of albumin on the crystallisation of CaOx in urine of South Africa's population groups has not been previously undertaken. While results for white subjects can be compared with studies from elsewhere in the world, the black population group is unique and the apparent immunity of this group provided the motivation for this study.

Following the successful isolation, purification and verification of urinary albumin from the two race groups, crystallisation experiments were undertaken to ascertain the effect of albumin on CaOx crystallisation kinetics, Coulter Counter measurements of particle volume-size distributions, [^{14}C]-oxalate deposition rates, zeta-potential measurements and crystal sedimentation rates. Previous studies on albumin have not adopted as rigorous a procedure as adopted in this study – multiple techniques were employed whereas previous studies used only one or two.

Data acquired by spectrophotometry, Coulter Counter and [^{14}C]-oxalate deposition were considered in concert and demonstrated that albumin from both race groups inhibits the rate of formation of CaOx crystals. These results are in harmony with recent findings (Edyvane et al., 1987; Grover et al., 1998; Chen et al., 2001). In BUF, WA was a stronger inhibitor of [^{14}C]-oxalate deposition than BA. However, the results for spectrophotometry and Coulter Counter indicated that BA was a superior inhibitor compared to WA. The discrepancy in these results was attributed to independent protein co-precipitation. In WUF, BA was found to be a more potent inhibitor of CaOx crystal formation.

Results on [^{14}C]-oxalate deposition rates in the current study demonstrated that albumin, irrespective of its source, inhibited CaOx crystallisation. Interestingly, previous studies have referred to albumin's effect on CaOx crystal growth as modest or absent (Edyvane et al., 1987; Worcester et al., 1988; Grover et al., 1998; Chen et al., 2001). The reason why the results of the present study do not agree with that reported in literature is that different assay systems were used.

Several investigators (Edyvane et al., 1987; Ryall et al., 1991; Hess et al., 1995; Grover et al., 1998; Cerini et al., 1999) have reported that crystals that form in the presence of albumin are smaller and more numerous than those in control solutions. It is worth noting that in all the above mentioned studies, commercial human serum albumin was used in real urine or in inorganic solution. The advantage of the present study over those mentioned

above is that *urinary* albumin was used in *real* urine. While there may not be any compositional, structural and conformational differences between human serum albumin (HSA) and urinary albumin, the different solutions might explain why the formation of smaller crystals in greater numbers, as described above, was not observed in the present study.

Urine composition was also found to be important. Albumin from both race groups when added to BUF formed fewer crystals and their total volume was less than those crystals which formed in WUF, suggesting a synergistic relationship between protein inhibitory performance and urine environment.

Another interesting observation was the formation of predominantly COD crystals in urine from healthy black subjects. Urine from healthy white subjects contained a mixture of COM and COD crystals. This was interpreted as a favourable response, as production of COD might afford the black group protection because COD crystals are less likely to bind to the renal epithelial cells and be retained in the kidney.

An important aim of this thesis was to access whether the inhibition of aggregation by albumin from black subjects was superior to albumin from white subjects. Indeed results from the sedimentation and zeta-potential experiments have shown this to be so. In the presence of BA, COM crystals carry a more negative charge than in the presence of WA. Therefore, BA will exert stronger repulsive forces and is likely to be a superior inhibitor of crystal aggregation than WA. These results were confirmed by sedimentation experiments and agree with results published in the literature for albumin (Hess et al., 1989). These results are significant because crystal aggregation is considered to be the most important mechanism necessary for stone formation. The present study demonstrated that BA is superior to WA with respect to inhibition of this mechanism.

Therefore, the results of this thesis provide compelling evidence to suggest that it is reasonable to speculate that albumin may play a contributory role to the overall immunity of the black population group in South Africa to urolithiasis.

Bibliography

(1) Ackermann D, Baumann J, Futterlieb A, Zingg E: Influence of Calcium Content in Mineral Water on Chemistry Crystallisation Conditions in Urine of Calcium Stone Formers. *Eur Urol* 14:305-308, 1988

(2) Aggarwal S, Tandon C, Forouzandeh M, Singla S, Kiran R, Jethi R: Role of Biomolecules from Human Renal Stone Matrix on COM Crystal Growth. *Molecular and Cellular Biochemistry* 210:109-119, 2000

(3) Allie S, Rodgers A: Effects of Calcium Carbonate, Magnesium Oxide and Sodium Citrate Bicarbonate Health Supplements on the Urinary Risk Factors for Kidney Stone Formation. *Clin Chem Lab Med* 1:39-45, 2003

(4) Asplin J, Bushinsky K, Singharetnam W, Parks J, Coe F: Relationships between Supersaturation and Crystal Inhibition in Hypercalciuric Rats. *Kidney Int* 51:640-645, 1997

(5) Asplin J, Deganello S, Nakagawa Y: Evidence that Nephrocalcin and Urine Inhibit Nucleation of Calcium Oxalate Monohydrate Crystals. *Am J Physiol.* 261:F824-830, 1991

(6) Asplin J, Hoyer J, Gillespie C. Coe F: Uropontin (UP) Inhibits Aggregation of Calcium Oxalate Monohydrate (COM) Crystals. *J Am Soc Nephrol* 6:941, 1995

(7) Atmani F, Khan S: Characterisation of Uronic-Acid-Rich Inhibitor of Calcium Oxalate Crystallisation Isolated from Rat Urine. *Urol Res* 23:95-101, 1995

(8) Atmani F, Khan S: Quantification of Proteins Extracted from Calcium Oxalate and Calcium Phosphate Crystals Induced in vitro in the Urine of Healthy Controls and Stone-Forming Patients. *Urol Int* 68:54-59, 2002

(9) Atmani F, Khan S: Role of Urinary Bikunin in the Inhibition of Calcium Oxalate Crystallisation. *J Am Soc Nephrol* 10(suppl 14):S385-8, 1999

(10) Atmani F, Lacour B, Drueke T, Daudon M: Isolation and Purification of a New Glycoprotein from Human Urine Inhibiting Calcium Oxalate Crystallisation. *Urol Res* 21:61-66, 1993

(11) Atmani F, Lacour B, Strecker G, Parvy P, Drueke T, Daudon M: Molecular Characteristics of Uronic-Acid-Rich Protein, A Strong Inhibitor of Calcium Oxalate Crystallisation in vitro. *Urol Res* 22:257-260, 1994

(12) Atmani F, Mizon J, Khan S: Identification of Uronic-Acid-Rich Protein as Urinary Bikunin, a Light Chain of Inter-α-trypsin-inhibitor. *Eur J Biochem* 236:984-990, 1996

(13) Atmani F, Opalko F, Khan S: Association of Urinary Macromolecules with Calcium Oxalate Crystals Induced in vitro in Normal Human and Rat Urine. *Urol Res* 24:45-50, 1996

(14) Atmani F, Opaldo J, Khan S: Association of Urinary Macromolecules with Calcium Oxalate Crystals Induced *in vitro* in Normal Human and Rat Urine. *Urol Res* 24:45-50, 1996

(15) Bateson E: Renal Tract Calculi and Climate. *Med. J. Aust.* 2:111-113, 1973

(16) Baumann J, Affolter B, Brenneisen J, Siegrist H: Measurement of Metastability, Growth and Aggregation of Calcium Oxalate in Native Urine. *Urol Int* 59:214-220, 1997

(17) Baumann J et al: Problems Related to the Measurement of Crystallisation conditions in Whole Urine. *Urol Res* 17:143-151, 1989

(18) Baumann J, Affolter B, Caprez U, Lauper D, Maier F, Siegrist H, Zysset T: Nucleation and Aggregation of Calcium Oxalate in Whole Urine; Spectrophotometric Sedimentation Analysis: A New Approach to Study the Aggregation of Calcium Oxalate Dihydrate. *Urol Res* 28:147-154, 2000

(19) Bergsland K, Kinder J, Asplin J, Coe B, Coe F: Influence of Gender and Age on Calcium Oxalate Crystal Growth Inhibition by Urine from Relatives of Stone Forming Patients. *J Urol* 167:2372-2376, 2002
(20) Berland Y, Olmer M, Boistelle R, Barlatier A: Respective Effect of Calcium and Oxalate on Calcium Oxalate Crystallisation in the Urine. *Nephrology* 5:175-179, 1984
(21) Binette J, Binette M, Gawinowicz, Kendrick N: Urinary Stone Proteins: An Update. *Scanning Microscopy* 10:509-518, 1996
(22) Boeve E, Cao L, de Bruijn W, Robertson W, Romijn J, Schroder F: Zeta Potential Distribution on Calcium Oxalate Crystal and Tamm-Horsfall Protein Surface Analysed with Doppler Electrophoretic Light Scattering. J Urol 152:531-536, 1994
(23) Borghi L, Meschi T, Amato F, Novarini A Romanelli A, Cigala F: Hot Occupation and Nephrolithiasis. *J Urol* 150:1757-1760, 1993
(24) Borghi L, Meschi T, Scianchi T, Briganti A, Guerra A, Allegri J, Novarini A: Urine Volume: Stone Risk Factor and Preventive Measure. *Nephron* 81 (Suppl. 1):31-37, 1999
(25) Borghi L, Schianchi T, Meschi T, Guerra A, Allegri F, Maggiore U, Novarini A: Comparison of Two Diets for the Prevention of Recurrent Stones in Idiopathic Hypercalciuria. *N Engl J Med* 346:77-84, 2002
(26) Boyce W: Organic Matrix of Human Urinary Concretions. *Am J Med* 45:673-683, 1968
(27) Boyce W, Garvey F: The amount and nature of the Organic Matrix of Urinary Calculi. *J Urol* 76:213, 1956
(28) Boyce W, King J, Fielden M: Total non-dialysable Solids (TNDS) in human urine. XIII. Immunological Detection of a Component Peculiar to Renal Calculus Matrix and to Urine of Calculus Patients. *J Clin Invest.* 41:1180-1189, 1962
(29) Bradford M: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilising the Principle of Protein – Dye Binding. *Analytical Biochemistry* 72:248-254, 1976
(30) Breslau N, Padalino P, KOK D, Kim Y, PaK C; Physicochemical Effects of a New Slow-Release Potassium Phosphate Preparation (UroPhos-K) in Absorptive Hypercalciuria. *J Bone Miner Res* 10(3):394-400.
(31) Brien G, Schubert G, Bick C: 10,000 Analyses of Urinary Calculi Using X-ray Diffraction and Polarising Microscopy. *Eur Urol* 8:251-256, 1982
(32) Brinkley L, McGuire J, Gregory J, Pak C: Bioavailability of Oxalate in Food. *Urology* 17:534-538, 1981
(33) Brown C, Ackermann D, Purich D: EQUIL 93: A Tool for Experimental and Clinical Urolithiasis. *Urol Res* 22:119-126, 1994
(34) Butler W: The Nature and Significance of Osteopontin. *Connect Tissue Res* 23:123-136, 1989
(35) Cao L, Boeve E, Schroder F, Robertson W, Ketelaars G, de Bruijn W: The Effect of Two New Synthetic Glycosaminoglycans (G871, G872) on the Zeta Potential of Calcium Oxalate Crystals and on Growth and Agglomeration. *J Urol* 147:1643-1646, 1992
(36) Cao L, Deng G, Boeve E, de Bruijn W, de Water R, Verkoelen C, Romijn J, Schroder F: Zeta Potential Measurement and Particle Size Analysis for a Better Understanding of Urinary Inhibitors of Calcium Oxalate Crystallisation. *Scanning Microsc.* 10:401-414, 1996
(37) Cerini C, Geider S, Dussol B, Hennequin C, Daudon, Veesler S, Nitsche S, Boistelle R, Berthezene P, Dupuy P, Vazi A, Berland Y, Dagorn J-C Verdier J-M:

Nucleation of Calcium Oxalate Crystals by Albumin: Involvement in the Prevention of Stone Formation. *Kidney Int* 55:1776-1786, 1999

(38) Chan V, Li M: Quantitative Analysis on the Localisation of Chondroitin Sulphate Proteoglycan in Renal Tissues of Patients with Calcium Nephrolithiasis. *Urol Res* 26:271-274, 1998

(39) Chang L, Lin H, Chen W: The Reappraisal of Nephrocalcin – its role in the Inhibition of Calcium Oxalate Crystal Growth and Interaction with Divalent Metal Ions. *Urol Res* 29(2):89-93, 2001

(40) Chen W, Lin H, Chen H, Shih C, Li C: Effects of Tamm-Horsfall Protein and Albumin on Calcium Oxalate Crystallisation and Importance of Sialic Acids. Molecular Urology 5:1-5

(41) Coe F: Clinical Stone Disease, in *Nephrolithiasis*, edited by Coe F, Brenner B, Stein J, 1980, pp 1-12

(42) Coe F, Parks J, Nakagawa Y: Protein Inhibitors of Crystallisation. S*eminars in Nephrology*. 11:98-109, 1997

(43) Coe F, Parks J, Nakagawa Y: Protein Inhibitors of Crystallisation; *Seminars in Nephrology* 11:98-109, 1991

(44) Coe F, Parks J, Webb D: Stone-Formin Potential of Milk or Calcium-Fortified Orange Juice in Idiopathic Hypercalciuric Adults. *Kidney Int* 41:139-142, 1992

(45) Conte A, Roca Pk Genestar C, Grases F: The Relationship Between Orthophosphate and Pyrophosphate in Normal Subjects and in Patients with Urolithiasis. *Urol Res* 17(3):173-175, 1989

(46) Craig T, Rodgers A, Brandt W: Comparison of Tamm Horsfoll Mucoprotein (THM) in normal and stone-forming Caucasian and African males in South Africa. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 119-120

(47) Craig T, Rodgers A, Brandt W: Inhibition of Calcium Oxalate Crystallisation by Tamm Horsfall Mucoprotein (THM) From Two Different Population Groups. *Eurolithiasis* (9th European Symposium on Urolithiasis), Edited by Kok D, Romijn H, Verhagen P, Verkoelen C, 2001 pp 205-206

(48) Cramer W: Experimental Production of Kidney Lesions by Diet. *Lancet* II:174-175, 1932

(49) Crawford J, Crematy E, Alexander A: The Effect of Natural and Synthetic Polyelectrolytes on the Crystallisation of Calcium Oxalate. *Aust J Chem* 21:1067-1072, 1968

(50) Curhan G: Epidemiologic Evidence for the Role of Oxalate in Idiopathic Nephrolithiasis. *J Endourol* 13:629-631, 1999

(51) Curhan G, Willett W, Rimm E, Spiegelman D, Stampfer M: Prospective Study of Beverage Use and the Risk of Kidney Stones. *Am J Epidemiol* 143:240-247, 1996

(52) Curhan G, Willet W, Rimm E, Stampfer M: A Prospective Study of Dietary Calcium and Other Nutrients and the Risk of Symptomatic Kidney Stones. *N Engl J Med* 328:833, 1993

(53) Curhan G, Willet W, Speizer F, Spiegelman D, Stampfer M: Comparison of Dietary Calcium With Supplemental Calcium and Other Nutrients as Factors Affecting the Risk for Kidney Stones in Women. *Ann Int Med* 126:497, 1997

(54) Curhan G, Willet W, Speizer F, Stampfer M: Beverage Use and Risk for Kidney Stones in Women. *Ann Intern Med* 128:534-540, 1998

(55) Daudon M, Donsimoni R, Hennequin C, Fellahi S, Le Moel G: Sex- and Age-Related Composition of 10,617 Calculi Analysed by Infrared. *Urol Res* 23:319-326, 1995

(56) Davidson A: *Renal Diseases*. Billing and Sons Ltd, London pp3, 1981

(57) Dawson C, Grover P, Kanellos J, Pham H, Kupczyk G, Oates A, Ryall R: Inter-Alpha-Inhibitor in Calcium Stones. Clin Sci (Lond). 95(2)187-193, 1998
(58) Deganello S: Interaction between Nephrocalcin and Calcium Oxalate Monohydrate: A Structural Study. *Calcif Tissue Int* 48:421-428, 1991
(59) de O G Mendocana, Martini L, Baxmann A, Nishiura J, Cuppari L, Sigulem D, Heilberg I: Effect of Oxalate Load on Urinary Oxalate Excretion in Calcium Stone Formers. *J Ren Nutr* 13:39-46, 2003
(60) Desmars J, Tawashi R: Dissolution and Growth of Calcium Oxalate Monohydrate. Effect of Magnesium and pH. *Biochim. Biophys. Acta* 313:256, 1973
(61) Doremus R, Teich S, Silvis P: Crystallisation of Calcium Oxalate from Synthetic Urine. *Invest Urol* 15:469-472, 1978
(62) Doyle I, Marshall V, Dawson C, Ryall R: Calcium Oxalate Crystal Matrix Extract: The Most Potent Macromolecular Inhibitor of Crystal Growth and Aggregation Yet Tested in Undiluted Human Urine *In Vitro*. *Urol Res* 23:53-62, 1995
(63) Doyle I, Ryall R, Marshall V: Inclusion of Proteins into Calcium Oxalate Crystals Precipitated from Urine: A Highly Selective Phenomenon. *Clin Chem* 37:1589-1594, 1991
(64) Durrbaum D, Rodgers A, Sturrock E: A Study of Crystal Matrix Extract and Urinary Prothrombin Fragment 1 from a Stone-Prone and Stone-Free Population. *Urol Res* 29:83-88, 2001
(65) Durrbaum D, Rodgers A, Sturrock E: Urinary Prothrombin Fragment 1 in South Africa: Black or White. *Eurolithiasis* (9th European Symposium on Urolithiasis), Edited by Kok D, Romijn H, Verhagen P, Verkoelen C, 2001 pp 205-206
(66) Durrbaum D, Rodgers A, Sturrock E: Characterisation and Preliminary Inhibitory Study of Urinary Prothrombin Fragment 1 (UPTF1) from two Population Groups in South Africa. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 153-155
(67) Dussol B, Geider S, Lilova A, Leonetti F, Dupuy P, Daudon, Berland Y, Dagorn J-C, Verdier J-M: Analysis of the Soluble Organic Matrix of Five Morphologically Different Kidney Stones. *Urol Res* 23:45-51, 1995
(68) Ebisono S, Nishihata M, Inagaki T, Umehara M, Kohjimoto Y: Bikunin Prevents Adhesion of Calcium Oxalate Crystals to Renal Tubular Cells in Human Urine. *J Am Soc Nephrol* 10(suppl 14):S436-40, 1999
(69) Edyvane K, Ryall R, Marshall V: The Influence of Serum and Serum Proteins on Calcium Oxalate Crystal Growth and Aggregation. *Clin. Chim. Acta* 157:81-88, 1986
(70) Edyvane K, Ryall R, Mazzachi R, Marshall V: The Effect of Serum on the Crystallisation of Calcium Oxalate in Whole Human Urine: Inhibition Disguised as Apparent Promotion. *Urol Res* 15:87-92, 1987
(71) Elliot J: Structure and Composition of Urinary Calculi. *J Urol* 109:82, 1973
(72) Elliot J, Rabinowitz I: Calcium Oxalate Crystalluria: Crystal Size in Urine. *J Urol* 121:324-327, 1980
(73) Ettinger B, Pak C, Citron J, Thomas C, Adams-Huet B, Vangessel A: Potassium-Magnesium Citrate is an Effective Prophylaxis against Recurrent Calcium Oxalate Nephrolithiasis. *J Urol* 158:2069-2073, 1997
(74) Fan J, Chandhoke P, Grampas S: Role of Sex Hormones in Experimental Calcium Oxalate Nephrolithiasis. *J Am Soc Nephrol* 10 (suppl 14):S56-59, 1999
(75) Finch A, Kasidas G, Rose G: Urine Composition in Normal Subjects after Oral Ingestion of Oxalate-Rich Foods. *Clin. Sci.* 60:411-418, 1981
(76) Finlayson B: Physicochemical Aspects of Urolithiasis. *Kindney Int* 13:344-360, 1978

(77) Finlayson B, Khan S, Hackett R: Mechanism of Stone Formation: an Overview. *Scanning Electron Microsc* III:1419-11425, 1984
(78) Finlayson B, Reid F: The expectation of Free and Fixed Particles in Urinary Stone Disease. *Invest Urol* 15:422-428, 1978
(79) Finlayson B, Vermeulen C, Stewart E: Stone Matrix and Mucoprotein from Urine. *J Urol* 86:355-363 , 1961
(80) Fleisch H: Inhibitors and Promoters of Stone Formation. *Kidney Int* 13:361-371, 1978
(81) Fleisch H, Bisaz S: Mechanism of Calcification: Inhibitory Role of Pyrophosphate. *Nature* 195:911, 1962
(82) Furedi-Milhofer H, Tunik L, Filipovic-Vincekovic N, Skrtic D, Babic-Ivancic V, Garti N: Induction of Crystallisation of Calcium Oxalate Dihydrate in Micellar Solutions of Anionic Surfactants. *Scanning Microsc.* 9:1060-1069, 1995
(83) Garilevich B, Avdeichuk I: The Characteristics of Urolithiasis in Flight Personnel and the Treatment Results Using a Method of Extracorporeal Shockwave Lithotripsy on the Russian Lithotripter Urat-P2. *Aviakosm Ekolog Med* 29:46-47, 1995
(84) Giannini S, Nobile M, Sartori L, Carbonare L, Ciuffreda M, Corro P, D'Angelo A, Calo L, Crepaldi G: Acute Effects of Moderate Dietary Protein Restriction in Patients with Idiopathic Hypercalciuria and Calcium Nephrolithiasis. *Am J Clin Nutr.* 69:267-271, 1999
(85) Gill W, Silver M, Roma M: Supersaturation Levels and Crystallisation Rates of Calcium Oxalate from Urines of Normal Humans and Stone Formers Determined by a 14C-Oxalate Technique. *Invest Urol* 12:203-209, 1974
(86) Goldfarb S: Diet and Nephrolithiasis. *Annu. Rev. Med* 45:235-243, 1994
(87) Goldfarb S, Asplin J: Effect of Grapefruit Juice on Urinary Lithogenicity. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 317-8
(88) Grases F, Conte A, Gill J: Simple Method for the Study of Heterogeneous Nucleation in Calcium Oxalate Urolithiasis. *BJU* 61:468-473, 1988
(89) Grases F, Ramis M, Costa-Bauza A: Effects of Phytate and Pyrophosphate on Brushite and Hydroxapatite Crystallisation. Comparison with the Action of Other Polyphosphates. *Urol Res* 28(2):136-140, 2000
(90) Grover K, Moritz R, Simpson R, Ryall R: Inhibition of Growth and Aggregation of Calcium Oxalate Crystals *In Vitro* – A Comparison of Four Human Proteins. *Eur. J Biochem* 253:637-644, 1998
(91) Grover P, Ryall R, Marshall V: Does Tamm-Horsfall Mucoprotein Inhibit or Promote Calcium Oxalate Crystallisation in Human Urine? *Clin. Chim. Acta* 190:223-238, 1990
(92) Hall P: Preventing Kidney Stones: Calcium Restriction not Warranted. *Cleve Clin J Med* 69:855-858, 2002
(93) Hallson P, Rose A: Seasonal Variations in Urinary Crystals. *BJU* 49:277-284, 1977
(94) Hallson P, Rose G, Sulaiman S: Magnesium Reduces Calcium Oxalate Crystal Formation in Human Whole Urine. *Clinical Science* 62:17-19, 1982
(95) Hallson P, Rose G, Sulaiman S: Pyrophosphate does not Influence the Calcium Oxalate or Calcium Phosphate Crystal Formation in Whole Urine. *Urol Res* 11:151-154, 1983
(96) Hallson P, Rose G, Sulaiman S: Raising Urinary Citrate Lowers Calcium Oxalate and Calcium Phosphate Crystal Formation in Whole Urine. *Urol Int* 38:179-181, 1983

(97) Hammar M, Berg G, Larsson L, Tiselius H, Varenhorst E: Endocrine Changes and Urinary Citrate Excretion. *Scand J Urol Nephrol* 21:51-53, 1987
(98) Heller H: The Role of Calcium in the Prevention of Kidney Stones. J *Am Coll Nutr* 18:373S, 1999
(99) Heller H, Sakhaee K, Moe O, Pak C: Etiological Role of Estrogen Status in Renal Stone Formation. *J Urol* 168:1923-1927, 2002
(100) Hemmingsen L, Skaarup P: The 24-hour Excretion of Plasma Proteins in the Urine of Apparently Healthy Subjects. *Scand J Clin Lab Invest* 35:347-353, 1975
(101) Hess B: Tamm-Horsfall Glycoprotein and Calcium Nephrolithiasis. *Miner Electrolyte Metab* 20:393-398, 1994
(102) Hess B: Tamm-Horsfall Glycoprotein – Inhibitor of Promoter of Calcium Oxalate Monohydrate Crystallisation Processes *Urol Res* 20:83-86, 1992
(103) Hess B, Kok D: Nucleation, Growth and Aggregation of Stone-Forming Crystals in Kidney Stones: Medical and Surgical Management edited by Coe F, Favus M, Pak C, Parks J, Preminger G, 1996, pp 3-32
(104) Hess B, Mauron H, Ackermann D, Jaeger P: Effects of a 'Common Sense Diet' on Urinary Composition and Supersaturation in Patients with Idiopathic Calcium Urolithiasis. *Eur Urol.* 36:136-143, 1999
(105) Hess B, Meindhardt U, Zipperle L, Giovanoli R, Jagger P: Simultaneous Measurements of Calcium Oxalate Crystal Nucleation and Aggregation: Impact of Various Modifiers. *Urol Res* 23:231-238, 1995
(106) Hess B, Nakagawa Y, Coe F: Inhibition of Calcium Oxalate Monohydrate Crystal Aggregation by Urine Proteins. *Am J Physiol* 257:F99-F106,1989
(107) Hess B, Ryall R, Kavanagh J, Khan S, Kok D, Rodgers A, Tiselius H: Methods for Measuring Crystallisation in Urolithiasis Research: Why, How and When? *European Urology* 40:220-230, 2001
(108) Hesse A, Siener R, Heynck H, Jahnen A: The Influence of Dietary Factors on the Risk of Urinary Stone Formation. *Scanning Microsc* 7:1119-1128, 1993
(109) Hirvonen T, Pietinen P, VirtanenM, Albanes D, Virtamo J: Nutrient Intake and Use of Beverages and the Risk of Kidney Stones among Male Smokers. *Am J Epidemiol* 150:187-194, 1999
(110) Hodgkinson A: Citric Acid Excretion in Normal Adults and in Patients with Renal Calculus. *Clin Sci* 23:203-212, 1962
(111) Honow R, Laube N, Schneider A, Keβler T, Hesse A: Influence of Grapefruit Juice on Urinary Parameters and Risk of Crystallisation in Comparison with Other Juices. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 319-320
(112) Hosking K, Erickson S, Van Den Berg C, Wilson D, Smith L: The Stone Clinic Effect in Patients with Idiopathic Calcium Urolithiasis. *J Urol* 130:1115, 1983
(113) Hosking D, Wilson J, Liedtke R, Smith L, Wilson D: Urinary Citrate Excretion in Normal Persons and Patients with Idiopathic Calcium Urolithiasis. *J Lab Clin Med* 106:682-689, 1985
(114) Hoyer J: Uropontin in Urinary Calcium Stone Formation. *Miner Electrolyte Metab* 20:385-392, 1994
(115) Hunt J, McGiven A, Grouvsky A, Lynn K, Taylor M: Affinity-purified Antibodies of Defined Specificity for use in a Solid-phase Microplate Radioimmunoassay of Human Tamm-Horsfall Glycoprotein in Urine. *Biochem J* 227:957-963, 1994
(116) Iguchi M, Takamura C, Umekawa T, Kurita T, Kohri K: Inhibitory Effects of Female Sex Hormones on Urinary Stone Formation in Rats. *Kidney Int* 56:479-485, 1999

(117) Iguchi M, Takamura C, Umekawa T, Kurita T, Kohri K: The Inhibitory Effect of Female Sex Hormones on Urinary Stone Formation in Rats. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 164-165
(118) Iwata H, Lio S, Nishio S, Takeuchi M: Architecture of Mixed Calcium Oxalate Dihydrate and Monohydrate Stones. *Scanning Microsc.* 6:231-238, 1992
(119) Johansson G, Backman U, Danielson B, Fellstrom B, Ljunghall S, Wikstrom B: Biochemical and Clinical Effects of the Prophylactic Treatment of Renal Calcium Stones with Magnesium Hydroxide. *J Urol* 124:770-774, 1980
(120) Jones W, Resnick M: The Characterisation of Soluble Matrix Proteins in Selected Human Renal Calculi Using Two-Dimensional Polyacrylamide Gel Electrophoresis. *J Urol* 144:1010-1014, 1990
(121) Kavanagh J: Supersaturation: What Role Does it Play in Calcific Stone Formation? in *Urolithiasis 2000* edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 41-46
(122) Kavanagh J, Nishio S, Garside J, Blacklock N: Crystallisation Kinetics of Calcium Oxalate in Fresh Minimally Diluted Urine: Comparison of Recurrent Stone Formers and Healthy Controls in a Continuous Mixed Suspension Mixed Product Removal Crystalliser. *J Urol* 149:614-617, 1993
(123) Khan S: Calcium Oxalate Crystal Interaction with Renal Tubular Epithelium, Mechanism of Crystal Adhesion and its Impact on Stone Development. *Urol Res* 23:71-79, 1995
(124) Khan S, Hackett R: Crystal-matrix relationships in experimentally induced calcium oxalate monohydrate crystals, an ultrastructural study. *Calcific Tissue Int* 41:157-163
(125) Khan S, Shevock P, Hackett R: Presence of Lipids in Urinary Stones: Results of Preliminary Studies. *Calif Tissue Int* 42:91-96, 1988
(126) Knight E, Stampfer M, Rimm E, Hankinson S, Curhan G: Moderate Alcohol Intake and Renal Function Decline in Women: A Prospective Study. *Nephrol Dial Transplant* 18:1549-1554, 2003
(127) Kok D, Iestra J, Doorenbos C, Papapoulos S: The Effects of Dietary Excess in Animal Protein and in Sodium on the Composition and the Crystallisation Kinetics of Calcium Oxalate Monohydrate in Urines of Healthy Men. *J Clin Endocrinol Metab* 71:861-867, 1990
(128) Kok D, Khan S: Calcium Oxalate Nephrolithiasis, a Free or Fixed Particle Disease. *Kidney Int* 47:847-854, 1994
(129) Kok D, Schell-Feith E: Risk Factors for Crystallisation in the Nephron: the Role of Renal Development. J Am Soc Nephrol 14:S364-S370, 1999
(130) Koul H, Menon M, Chaturvedi L, Koul S, Sekhon A, Bhandari A, Huang M: COM Crystals Activate the p38 mitogen-activated kinase signal transduction pathway in renal epithelial cells. *J Biol Chem* 277:36845-52, 2002
(131) Kragh-Hansen: Molecular Aspects of Ligand Binding to Serum Albumin. *Pharmacological Reviews* 33:17-53, 1981
(132) Kumar S, Muchmore A: Tamm-Horsfall Protein – Uromodulin (1950-1990). *Kidney Int* 37:1395-1401, 1990
(133) Larsson L, Tiselius H: Hyperoxaluria. *Miner. Electrolyte. Metab.* 13:242-250, 1987
(134) Levi M, McDonald A, Preisig P, Apern R: Chronic K+ Depletion Stimulates Rat Renal Brush-Border Membrane Na-Citrate Cotransporter. *Am J Physiol* 261:F767-F773, 1991
(135) Levine B, Rodman J, Wienerman S, Bockman R, Lane J: Effect of Calcium Citrate Supplementation on Urinary Calcium Oxalate Saturation in Female Stone Formers: Implications for Prevention of Osteoporosis. *Am J Clin Nutr* 60:592-596, 1994

(136) Lewandowski S, Rodgers A, Schloss I: The Influence of a High-oxalate/Low-Calcium Diet on Calcium Oxalate Renal Stone Risk Factors in Non-stone forming Black and White South African Subjects. *BJU* 87:307-311, 2001
(137) Lieske J, Leonard R, Toback F: Adhesion of Calcium Oxalate Monohydrate Crystals to Renal Epithelial Cells is Inhibited by Specific Anions. *Am J Physiol* 268: F604-F612, 1995
(138) Lieske J, Walsh-Reitz M, Toback F: Calcium Oxalate Monohydrate Crystals are Endocytosed by Renal Epithelial Cells and Induce Proliferation. *Am J Physiol* 262:F622-F630, 1992
(139) Ljunghall S: Incidence of Upper Urinary Tract Stones. *Min Electrolyte Metab* 13:220-227, 1987
(140) Ljunghall S, Hedstrand H: Epidemiology of Renal Stones in a Middle-Aged Male Population. *Acta Med Scand* 197:439-445, 1975
(141) Mandel N, Mandel G: Physicochemistry of Urinary Stones, in Renal *Stone Disease*, edited by Pak C, 1987, pp1-24
(142) Marangella M, Vitale C, Petrarulo M, Bagnis C, Bruno M, Ramello A: Renal Stones: From Metabolic to Physicochemical Abnormalities. How Useful are Inhibitors? *J Nephrol* 13(suppl. 3):S51-S60, 2000
(143) March J, Simonet B, Grases F: Determination of pyrophosphate in Renal Calculi and Urine by Means of an Enzymatic Method. *Clin Chim Acta.* 314(1-2):187-194, 2001
(144) Martini L, Wood R: Should Dietary Calcium and Protein be Restricted in Patients with Nephrolithiasis. *Nutr Rev* 58:111-117, 2000
(145) Maslamani S, Glenton P, Khan S: Changes in Urine Macromolecular Composition During Processing. *J Urol* 164:230-236
(146) Massey L, Kynast-Gales S: Diets with Either Beef or Plant Proteins Reduce Risk of Calcium Oxalate Precipitation in Patients with a History of Calcium Kidney Stones. *J Am Diet Asoc* 101:326-331, 2001
(147) Massey L, Roman-Smith H, Sutton R: Effect of Dietary Oxalate and Calcium on Urinary Oxalate and Risk of Formation of Calcium Oxalate Kidney Stones. *J Am Diet Assoc* 93:901, 1993
(148) Massey L, Roman-Smith H, Sutton R: Substituting Milk for Apple Juice does not Increase Kidney Stone Risk in Most Normocalciuric Adults who Form Calcium Oxalate Stones. *J Am Diet Assoc* 98:303-308, 1998
(149) McHarg T, Rodgers A, Charlton K: Influence of Cranberry Juice on the Urinary Risk Factors for Calcium Oxalate Kidney Stone Formation. *BJU* 92:765-768, 2003
(150) McQueen E, Engel G: Factors Determining the Aggregation of Urinary Mucoprotein. *J Clin Pathol* 19:392-396, 1966
(151) Menon M, Mahle C: Urinary Citrate Excretion in Patients with Renal Calculi. *J Urol* 129:1158-1160, 1983
(152) Messa P, Marangella M, Paganin L, Codardini M, Cruciatti A, Turrin D et al: Different Dietary Calcium Intake and Relative Supersaturation of Calcium Oxalate in the Urine of Patients Forming Renal Stones. *Clin Sci*, 93:257, 1997
(153) Meyers A, Whalley N, Zakolski W, Shar T: Chemical Composition of the Urine of the Normal Black and White Population, in *Urolithiasis 2*, edited by Ryall R, Bais R, Marshall V, Rofe A, Smith L, Walker V, 1994, pp 422
(154) Meyer J: Physicochemistry of Stone formation in *UROLITHIASIS – A Medical and Surgical Reference* edited by Resnick and Pak, 1990, pp 11-34
(155) Meyer J, Smith L: Growth of Calcium Oxalate Crystals. Inhibition by Natural Growth Inhibitors. *Invest Urol* 13:36-39, 1975

(156) Min He X, Carter D: Atomic Structure and Chemistry of Human Serum Albumin. *Nature* 358:209-215, 1992
(157) Moriyama M, Glenton P, Khan S: Expression of Inter-Alpha Inhibitor Related Proteins in Kidneys and Urine of Hyperoxaluric Rats. *J Urol* 165(5):1687-72, 2001
(158) Morse R, Resnick M: A New Approach to the Study of Urinary Macromolecules as a Participant in Calcium Oxalate Crystallisation. *J Urol* 139:869-873, 1998
(159) Morse R, Resnick M: A Study of the Incorporation of Urinary Macromolecules onto Crystals of Different Mineral Compositions. J Urol 141:641, 1989
(160) Morse R, Resnick M: Urinary Stone Matrix. J Urol 139:602-605, 1988
(161) Mustafi D, Nakagawa Y: Characterisation of (Ca2+)-binding sites in the Kidney Stone Inhibitor Glycoprotein Nephrocalcin usine Vanadyl ions: Different Metal Binding Properties in Strong and Weak Inhibitor Proteins Revealed by EPR and ENDOR. *Biochemistry* 35(47):14703-14709, 1996
(162) Nagata T, Todescan R, Goldberg H: Sulphation of Secreted Phosphoprotein I (SPPI, Osteopontin) is Associated with Mineralised Tissue Formation. *Biochem Biophys Res Commun* 165:234-340, 1989
(163) Najem G, Seebode J, Samady A, Feuerman M, Friedman L: Stressful Life Events and Risk of Symptomatic Kidney Stones. *Int J Epidemiology* 26:1017-1023, 1997
(164) Nakagawa Y: Properties and Function of Nephrocalcin: Mechanism of Kidney Stone Inhibition or Promotion. *Keio J Med.* 46(1):1-9, 1997
(165) Nakagawa Y, Abram V, Kezdy F: Purification and Characterisation of the Principal Inhibitor of Calcium Oxalate Monohydrate Crystal Growth in Human Urine. *J Biol Chem* 258:12594-12600, 1983
(166) Nakagawa Y, Abram V, Kezdy F, Kaiser E, Coe F: Purification and Characterisation of the Principal Inhibitor of Calcium Oxalate Monohydrate Crystal Growth in Human Urine. *J Biol. Chem* 258:12594-12600, 1983
(167) Nakagawa Y, Abram V, Parks J: Urine Glycoprotein Crystal Growth Inhibitors: Evidence for a Molecular Abnormality in Calcium Oxalate Nephrolithiasis. *J Clin Invest* 76:1455-1462, 1985
(168) Nakagawa Y, Netzer M, Coe F: Immunohistochemical Localisation of Nephrocalcin (NC) to Proximal Tubule and Thick Ascending Limb of Henle's Loop (TALH) of Human and Mouse Kidney (Abstract). *Kidney Int* 37:474
(169) Nguyen Q, Kalin A, Drouve U, Casez J, Jaeger P: Sensitivity to Meat Protein Intake and Hyperoxaluria in Idiopathic Calcium Stone Formers. *Kidney international* 59:2273-2281, 2001
(170) Nordin B, Need A, Morris H (eds): Metabolic Bone and Stone Disease, Churchill Livingston, Edingburg, 1993
(171) O'Brien M, Uhlemann I, McIntosh H: Urinary Pyrophosphate in Normal Subjects and in Stone Formers. *Can Med Assoc J* 96:100-103, 1967
(172) Ogawa Y: Impact of Sodium-Potassium Citrate on the Diurnal Variations in Urinary Calcium Oxalate and Calcium Phosphate Saturation Levels in Normal Individuals. *BJU* 73:136-141, 1994
(173) Pak C: Citrate and Renal Calculi: New Insights and Future Directions. *Am J Kidney Dis* 17:420-425, 1991
(174) Pak C: Citrate and Renal Calculi: An Update. *Miner Electrolyte Metab* 20:371-377, 1994
(175) Pak C, Ohata M, Holt K: Effect of Diphosphonate on Crystallisation of Calcium Oxalate in vitro. *Kidney Int.* 7:154-160, 1975
(176) Pak C, Fuller C: Idiopathic Hypocitraturic Calcium-Oxalate Nephrolithiasis Successfully Treated With Potassium Citrate. *Ann Int Med* 104:33-37, 1986

(177) Pak C, Fuller C, Sakhaee K, Preminger G, Britton F; Long-term Treatment of Calcium Nephrolithiasis with Potassium Citrate. *J Urol* 134:11-19, 1985
(178) Pak C, Holt K: Nucleation and Growth of Brushite and Calcium Oxalate in Urine of Stone Formers. *Metabolism* 25:665-673, 1976
(179) Pak C, Koenig K, Khan R, Haynes S, Padalino P: Physicochemical Action of Potassium-Magnesium Citrate in Nephrolithiasis. *J Bone Miner Res* 7:281-285, 1992
(180) Parivar J, Low R, Stoller M: The Influence of Diet on Urinary Stone Disease. *J Urol* 155:432-440, 1996
(181) Parks J, Coe F: A Urinary Calcium-Citrate Index for the Evaluation of Nephrolithiasis. *Kidney Int* 30:85-90, 1986
(182) Pin N, Ling N, Siang L: Dehydration From Outdoor Work and Urinary Stones in a Tropical Environment. *Occup Med (Lond)* 42 (1):30-32, 1992
(183) Prien E: The Riddle of Urinary Stone Disease. JAMA 216:503-507, 1971
(184) Punc J: Frequently Asked Questions About Albumin in *All About Transplantation and Donations*, 2003, pp1-2
(185) Ramello A, Vitale C, Marangella: Epidemiology of Nephrolithiasis. *J Nephrol.* 2000;13 (suppl. 3):S45-50
(186) Reis-Santos J: Age of First Stone Episode. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 375-378
(187) Roberts S, Resnick M: Glycosaminoglycans Content of Stone Matrix. *J Urol* 135:1078-1083, 1986
(188) Robertson W, Hughes H: Epidemiology of Urinary Stone Disease in Saudi Arabia, in *Urolithiasis 2*, edited by Ryall R, Bais R, Marshall V, Rofe A, Smith L, Walker V, 1994, pp 453-455
(189) Robertson W, Nordin B: Physico-Chemical Factors Governing Stone Formation in *Scientific Foundations of Urology* edited by William D, Chrisholm G, 1981, pp 254-267
(190) Robertson W, Peacock M: Calcium Oxalate Crystalluria and Inhibitors of Crystallisation in Recurrent Renal Stone-Formers. *Clin Sci* 43:499, 1972
(191) Robertson W, Peacock M: Review of Risk Factors in Calcium Oxalate Urolithiasis. *J Urol* 1:114-118, 1983
(192) Robertson W, Peacock M, Heyburn P, Bambach C: Risk Factors in Calcium Stone Disease, in *Urinary Calculus*, edited by Brockis and Finlayson, 1981, pp 265-273
(193) Robertson W, Peacock M, Heyburn J, Hanes F, Rutherford A, Clementson E, Swaminathan R, Clark P: Should Recurrent Calcium Oxalate Stone Formers become Vegetarians? *BJU* 51:427-431, 1979
(194) Robertson W, Peacock M, Hodgkinson A: Dietary Changes and the Incidence of Urinary Calculi in the UK between 1958 and 1976. *J Chron Dis* 32:469-476, 1979
(195) Robertson W, Peacock M, Heyburn P, Hanes F, Swaminathan R: The Risk of Calcium Stone Formation in Relation to Affluence and Dietary Animal Protein, in Urinary Calculus, edited by Brockis J and Finlayson B, 1981, pp 3-12
(196) Robertson W, Peacock M, Marshall R, Marshall D, Nordin B: Saturation-Inhibition Index as a Measure of the Risk of Calcium Oxalate Stone Formation in the Urinary Tract. *N Engl J Med* 294:249-252, 1976
(197) Robertson W, Peacock M, Nordin B: Inhibitors of the Growth and Aggregation of Calcium Oxalate Crystals In Vitro. *Clin Chim Acta* 43:31-37, 1973
(198) Rodgers A: Effect of Cola Consumption on Urinary Biochemical and Physicochemical Risk Factors Associated With Calcium Oxalate Urolithiasis. *Urol Res* 27:77-81, 1999

(199) Rodgers A: Effect of Mineral Water Containing Calcium and Magnesium on Calcium Oxalate Urolithiasis Risk Factors. *Urol Int* 58:93-99, 1997
(200) Rose A: Urinary Stones: Clinical and Laboratory aspects. MTP Press Limited, England pp 6-7, 1982
(201) Ruhn P, Taylor J, Hage D: Determination of Urinary Albumin Using High-Performance Immunoaffinity Chromatography and Flow Injection Analysis. *Anal Chem* 66:4265-4271, 1994
(202) Rushton H, Spector M: Effects of Magnesium Deficiency on Intratubular Calcium Oxalate Formation and Crystalluria in Hyperoxaluric Rats. *J Urol* 127:598-604, 1982
(203) Russell R, Hodgkinson A: The Urinary Excretion of Inorganic Pyrophosphate by Normal Subjects and Patients with Renal Calculus. *Clin Sci* 31:51-62, 1966
(204) Rodgers A, Lewandowski S: Effects of 5 Different Diets on Urinary Risk Factors for Calcium Oxalate Kidney Stone Formation: Evidence of Different Renal Handling Mechanisms in Different Race Groups. *J Urol* 168:931-936, 2002
(205) Ryall R: Glycosaminoglycans, Proteins and Stone Formation: Adult Themes and Child's Play. *Pediatr. Nephrol.* 10:656-666, 1996
(206) Ryall R: The Mystery of Macromolecules: Modulators, Matrix and Mineralisation. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000 pp 99-105
(207) Ryall R: Urinary Inhibitors of Calcium Oxalate Crystallisation and their Potential Role in Stone Formation. *World J Urol* 15:155-164, 1997
(208) Ryall R, Fleming D, Grover P, Chauvet M, Dean C, Marshall V: The Whole Truth: Intracrystalline Proteins and Calcium Oxalate Kidney Stones. *Molecular Urology* 4:391-402, 2000
(209) Ryall R, Grover P, Stapleton A, Barrell D, Tang Y, Moritz R, Simpson R: The Urinary F1 Activation Peptide of Human Prothrombin is a Potent Inhibitor of Calcium Oxalate Crystallisation in Undiluted Human Urine In Vitro. *Clin. Sci* 89:533-541, 1995
(210) Ryall R, Harnett R, Hibberd C Edyvane K, Marshall V: Effects of Chondroitin Sulphate, Human Serum Albumin and Tamm-Horsfall Mucoprotein on Calcium Oxalate Crystallisation in Undiluted Human Urine. *Urol Res* 19:181-188, 1991
(211) Ryall R, Harnett R, Marshall V: the Effect of Urine, Pyrophosphate, Citrate, Magnesium and Glycosaminoglycans on the Growth and Aggregation of Calcium Oxalate Crystals In Vitro. *Clin Chim Acta* 112:349-356, 1981
(212) Ryall R, Hibberd C, Marshall V: A Method for Studying Inhibitory Activity in Whole Urine. *Urol Res* 13:285-289, 1985
(213) Ryall R, Hibberd C, Mazzachi B, Marshall V: Inhibitory Activity of Whole Urine: A Comparison of Urine From Stone Formers and Healthy Subjects. *Clin Chim Acta* 154:59-68, 1986
(214) Sakhaee K, Baker S, Serwekh J, Poindexter J, Garcia-Hernandez P, Pak C: Limited Risk of Kidney Stone Formation During Long-term Calcium Citrate Supplementation in Nonstone Forming Subjects. *J Urol* 152:324-327, 1994
(215) Salier J-P: Inter-α-trypsin-inhibitor: Emergence of a Family within the Kunitz-type Protease Inhibitor Superfamily. *TIBS* 15:435-439, 1990
(216) Schwille P, Rumenapf G, Kohler R, Sorgel F: Effects of Acute Oral Sodium Potassium Citrate Load in Healthy Males – Outlook for Treatment of Patients With Calcium – Containing Renal Stones. *Urol Int* 42:81-88, 1987
(217) Schwille P, Schmiedl A, Herrmann U, Schwille R, Fink E, Manoharan M: Acute Oral Calcium-Sodium Citrate Load in Healthy Males. Effects on Acid-Base and

Mineral Metabolism, Oxalate and Other Risk Factors of Stone Formation in Urine. *Meth Find Exp Clin Pharmacol* 19:417-427, 1997

(218) Schwille P, Weippert J, Bausch W, Rumenapf G: Acute Oral Alkali Citrate Load in Healthy Humans- Response of Blood and Urinary Citrate, Mineral Metabolism, and Factors Related to Stone Formation. *Urol Res* 13:161-168, 1985

(219) Scurr D, Robertson W: Modifiers of Calcium Oxalate Crystallisation II. Studies on their Modes of Action in an Artificial Urine. *J Urol* 136:128-131, 1986

(220) Seltzer M, Low R, McDonald M, Shami G, Stoller M: Dietary Manipulation with Lemonade to Treat Hypocitraturic Calcium Nephrolithiasis. *J Urol* 156:907-909, 1996

(221) Senthil D, Subha K, Saravanan N, Varalakshmi P: Influence of Sodium Pentosan Polysulphate and Certain Inhibitors on Calcium Oxalate Crystal Growth. *Mol Cell Biochem.* 156:31-35, 1996

(222) Shigara H, Min W, VanDusen W, Clayman M, Miner D, Tarrell C, Sherbotie J, Foreman J, Przysiecki C, Neilson E, Hoyer J: Inhibition of Calcium Oxalate Crystal Growth in vitro by Uropontin: Another Member of the Aspartic-Acid Rich Protein Super Family. *Proc. Natl Acad. Sci.* USA 89: 426-430, 1992

(223) Shirane Y, Kagawa S: Scanning Electron Microscopic Study of the Effect of Citrate and Pyrophosphate on Calcium Oxalate Crystal Morphology. *J Urol* 150(6):1980-1983, 1993

(224) Shirane Y, Kurokawa Y, Sumiyoshi Y, Kagawa S: Morphological Effects of Glygosaminoglycans on Calcium Oxalate Monohydrate Crystals. *Scanning Microsc.* 9:1081-1088, 1995

(225) Shorr E, Bernheim A, Taussky H: The Relation of Urinary Citric Acid Excretion on the Menstrual Cycle and the Steroidal Reproductive Hormones. *Science* 95:606-607, 1942

(226) Shum D, Gohel M: Separate Effects of Urinary Chondroitin Sulphate and Heparan Sulphate upon the Crystallisation of Urinary Calcium Oxalate: Differences between Stone Formers and Normal Controls. *Clin Sci* 85:33-39

(227) Shuster J, Finlayson B, Scheaffer R, Sierakowski R, Zoltek J, Dzegede S: Water Hardness and Urinary Stone Disease. *J Urol* 128:422-425, 1982

(228) Sidhu H, Gupta R, Thind S, Nath R: Inhibition of Calcium Oxalate Monohydrate (COM) Crystal Growth by Pyrophosphate, citrate and Rat Urine. *Urol Res* 14(6):299-303, 1986

(229) Siener R, Hesse A: Influence of a Mixed and a Vegetarian Diet on Urinary Magnesium Excretion and Concentration. *British Journal of Nutrition* 73:783-790, 1995

(230) Siener R, Hesse A: The Role of Age in Calcium Oxalate Stone Formation. *Urolithiasis 2000*, edited by Rodgers A, Hibbert B, Hess B, Khan S, Preminger G, 2000, pp 422-423

(231) Sikiric M, Filipovic-Vincekovic N, Babic-Ivanci & cacute V, Vdovi & cacute N, Furedi-Milhofer H: Interactions in Calcium Oxalate Hydrate/Surfactant Systems. *J Colloid Interface Sci.* 212:384-389, 1999

(232) Smith L: Diet and Hyperoxaluria in the Syndrome of Idiopathic Calcium Oxalate Urolithiasis. *Am J Kidney Dis* 17:370-375, 1991

(233) Sorenson S, Hansen K, Bak S, Justesen S: An Unidentified Macromolecular Inhibitory Constituent of Calcium Oxalate Crystal Growth in Human Urine. *Urol Res* 18:373-379, 1990

(234) Soucie M, Thun M, Coates R, McClellan W, Austin H: Demographic and Geographic Variability of Kidney Stones in the United States. *Kidney Int* 46:893-899,1994

(235) Stapleton A, Ryall R: Blood Coagulation Proteins and Urolithiasis are Linked: Crystal Matrix Protein is the F1 activation Peptide of Human Prothrombin. *BJU* 75:712-719, 1995
(236) Stapleton A, Seymour A, Brennan J, Doyle I, Marshall V, Ryall R: The Immunohistochemical Distribution and Quantification of Crystal Matrix Protein. *Kidney Int* 44:817-824, 1993
(237) Stryer L: *Biochemistry*, WH Freeman and Co, New York, 1995
(238) Suzuki M, Kobayashi H, Kageyama S, Shibata K, Fujie M, Terao T: Excretion of Bikunin and its Fragments in the Urine of Patients with Renal Stones. *J Urol.* 166(1):268-274, 2001
(239) Sutor D, Scheidt S: Identification Standards for Human Urinary Calculus Components, Using Crystallographic Methods. *BJU* 40:22-28, 1968
(240) Suzuki K, Ryall R: The Effect of Heparan Sulphate on the Crystallisation of Calcium Oxalate in Undiluted, Ultrafiltered Human Urine. *BJU* 78:15-21
(241) Tang Y, Grover P, Moritz R, Simpson R, Ryall R: Is Nephrocalcin Related to the Urinary Derivative (Bikunin) of Inter-α-trypsin-inhibitor? *BJU* 75:425-430, 1995
(242) Tiselius H: Oxalate and Renal Stone Formation. *Scand J Urol Nephrol* (Suppl.) 53:135-134, 1980
(243) Tiselius H: The Effect of pH on the Urinary Inhibition of Calcium Oxalate Crystal Growth. *BJU* 53:470-474, 1981
(244) Tiselius H, Bek-Jensen H, Fornander A: Crystallisation Properties in Urine from Calcium Oxalate Stone Formers. *J Urol* 154:940-946, 1995
(245) Trinchieri A, Mandressi A, Luongo P, Longo G, Pisani E: The Influence of Diet on Urinary Risk Factors for Stones in Healthy Subjects and Idiopathic Renal Calcium Stone Formers. *BJU* 67:230-236, 1991
(246) Trinchieri A, Mandressi A, Luongo P, RoveraF, Longo G: Urinary Excretion of Citrate, Glycosaminoglycans, Magnesium and Zinc in Relation to Age and Sex in Normal Subjects and in Patients Who Form Calcium Stones. *Scand J Urol Nephrol* 26:379-386, 1992
(247) Vahlensieck W: Review: The Importance of Diet in Urinary Stones. *Urol Res* 14:283-288, 1986
(248) Wabner C, Pack C: A Simple Method for Quantitating the Propensity for Calcium Oxalate Crystallisation in Urine. *J Lithotr Stone Dis.* 3:255-259, 1991
(249) Wabner C, Pak C: Effect of Orange Juice Consumption on Urinary Stone Risk Factors. *J Urol* 149:1405-1408, 1993
(250) Webber D, Rodgers A, Sturrock E: Intracrystalline UPTF1 in Stone-Prone and Stone-Free South Africans: Does it Decide Their Fate? Urolithiasis (10th European Symposium on Urolithiasis), edited by Sarica K, Yagci F, Erbagci A, Inal Y, 2003, pp 83
(251) Webber D, Rodgers A, Sturrock E: Selective Inclusion of Proteins into Urinary Calcium Oxalate Crystals: Comparison between Stone-Prone and Stone-Free Population Groups. *JCG* 259:179-189, 2003
(252) Weiss G, Sluss P, Linke C: Changes in Urinary Magnesium, Citrate and Oxalate Levels Due to Cola Consumption. *Urology* 39:331-333, 1992
(253) Welshman S, McGeown M: Urinary Citrate Excretion in Stone-Formers and Normal Controls. *BJU* 48:7-11, 1976
(254) Wesson J, Worcester E, Kleiman J: Inhibitor Proteins in Urine Favour Formation of Calcium Oxalate Dihydrate (COD) Crystals, which have a Smaller Affinity for Renal Tubule Cells than Monohydrate (COM) Crystals. *J Am Soc Nephrol* 3:956, 1995

(255) Wesson J, Worcester E, Kleiman J: Role of Anionic Proteins in Kidney Stone Formation: Interaction Between Model Anionic Polypeptides and Calcium Oxalate Crystals. *J Urol* 163:1343-1348, 2000
(256) Wesson J, Worcester E, Wiessner J, Mandel N, Kleiman J: Control of Calcium Oxalate Crystal Structure and Cell Adherence by Urinary Macromolecules. *Kidney Int.* 53:952-957, 1998
(257) Whalley N, Meyers A, Martins M, Margolius: Long-term Effects of Potassium Citrate Therapy on the Formation of New Stones in Groups of Stone Formers with Hipocitraturia. *BJU* 78:10-14, 1996
(258) Whalley N, Martins M, van Dyk R, Meyers A: Lithogenic Risk Factors in Normal Black Volunteers and White Recurrent Stone Formers. *BJU* 84:243-248, 1999
(259) Whalley N, Moraes M, Shar T, Pretorius S, Meyers A: Lithogenic Risk Factors in the Urine of black and white subjects. *BJU* 82:758-790, 1998
(260) Wiggins R, Kshrisagar B, Kelsch R, Wilson B: Fragmentation and Polymeric Complexes of Albumin in Human Urine. *Clin Chim Acta* 149:155-163, 1985
(261) Wikstrom B, Danielson B, Ljunghall S McGuire M, Russell R: Urinary Pyrophosphate Excretion in Renal Stone Formers with Normal and Impaired Renal Function. *World J Urol* 1:150-154, 1983
(262) Worcester E: Inhibitors of Stone Formation. *Seminars in Nephrology*. 16:474-486, 1996
(263) Worcester E, Beshensky A: Osteopontin Inhibits Nucleation of Calcium Oxalate Crystals. *Ann N Y Acad Sci* 760:375-377, 1995
(264) Worcester E, Blumenthal S, Beshensky A: The Calcium Oxalate Crystal Growth Inhibitor Protein Produced by Mouse Kidney Cortical Cells in Culture is Osteopontin. *J Bone Miner Res* 7:1029-1036, 1992
(265) Worcester E, Nakagawa Y, Wabner C: Crystal Adsorption and Growth Slowing by Nephrocalcin, Albumin and Tamm-Horsfall Protein. *Am J Physiol* 255:F1197-1205, 1988
(266) Yamaguchi S, Yoshioka T, Utsonomiya M, Koide T, Wsafune M, Okuyama A, Sonoda T: Heparan Sulphate in the Stone Matrix and its Inhibitory Effect on Calcium Oxalate Crystallisation. *Urol Res* 21:187-192, 1993