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ORIGINAL PAPER

Inhibition of calcium oxalate crystallization by commercial human serum albumin and human urinary albumin isolated from two different race groups: evidence for possible molecular differences

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Abstract This study was undertaken to investigate the inhibitory activity of urine-derived (as opposed to serum-derived) albumin towards calcium oxalate crystallization and to compare the relative inhibitory strengths of this protein in subjects from South Africa's black and white population groups. Albumin was purified from the urines of 20 males in each race group using immunoaffinity chromatography. The purified proteins, as well as commercial human serum albumin were tested for their inhibition of calcium oxalate crystallization in ultra-filtered urines from both groups. Irrespective of its origin, albumin was found to be an inhibitor of calcium oxalate aggregation. Albumin derived from black subjects was superior to that from white subjects in this regard while urine-derived albumin was superior to that derived from serum. The composition of the urine in which the experiments were conducted influenced the inhibitory activity of the individual proteins. The different inhibitory activity of the proteins under identical conditions provides evidence that suggests molecular differences exist between them.

Keywords Albumin · Crystal inhibition · Calcium oxalate · Crystallization · Race groups · Urinary proteins

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Introduction

As part of our ongoing studies of the factors contributing to the rarity of urolithiasis in South Africa's black population, we have been investigating the role of urinary proteins in providing a protective mechanism in this race group. Thus far we have shown that urinary prothrombin fragment 1 [1] and Tamm Horsfall glycoprotein [2] from black subjects have superior inhibitory properties towards calcium oxalate (CaOx) crystallization than the same proteins from white subjects. Another urinary protein deserving of attention in this context is albumin. Several studies have demonstrated that this protein is a key constituent of CaOx crystals derived from urine [3, 4] and from pathological stones [5], prompting some researchers to propose that it plays a modulatory role [6] while others suggest that it may increase the adhesion of CaOx crystals to renal cells thereby increasing the chances of retention and stone formation [3, 4]. Another group suggests that albumin could act as a template for nucleation, growth or aggregation of crystals and that it could be one of the driving forces of the nucleation process [5]. These theories, based on the inclusion of albumin in CaOx crystals and stones, are speculative at best. On the other hand, studies involving the inhibitory or promotory effects of the protein towards CaOx crystallization would be expected to be more meaningful. Unfortunately, this is not the case. In vitro experiments involving both bovine [7] and human serum albumin (HSA) [8–14] in inorganic solutions are not in agreement with respect to the role of this protein in CaOx nucleation and growth. Some of these studies have shown that it has no effect on nucleation [7, 11] while others have demonstrated that it promotes this mech-

anism [13, 14]. Similarly, while one study has shown minor inhibition towards growth [8], another could not find any effect [12]. However, studies are unanimous in proclaiming that albumin is an inhibitor of CaOx crystal aggregation [7–9, 11]. Interestingly, a study involving commercial HSA in real urine found hardly any effect on aggregation [10], while another study in an inorganic solution, but involving albumin derived from human urine, supported the finding of aggregation inhibition [13].

Based on albumin's reputation as an inhibitor of CaOx crystal aggregation and on the paucity of experiments in which urine-derived albumin has been studied in real urine, we undertook to investigate these and other effects in subjects from South Africa's two population groups.

Materials and methods

Protein isolation

Sample preparation

Two different methods of sample preparation for immunoaffinity chromatography were used—urine concentration [13] and inclusion of proteins in CaOx crystals [4]—as it was not obvious from the literature whether one was superior to the other. For the former, 24 h 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 CaOx precipitation. The absence of blood and infection from individual samples was confirmed using Combur 10 urinalysis test strips (Boehringer Mannheim). Urines were filtered with a pre-filter (Macherey-Nagel), followed by a 0.42 μm Millipore filter (Millipore Corporation, Bedford) and were 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. The concentrate was dialysed extensively for 2 days against distilled water at 4°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 and 240 ml of PBS, respectively.

Crystal matrix extract (CME)

About 24 h urines were collected from two healthy black and seven healthy white male subjects and were tested for blood and infection as previously described, and were then pooled. (The number of white subjects was greater than that of black subjects because CaOx crystals induced in urines from the former group were required for optimisation experiments described in the demineralisation paragraph below). A modified version of the method reported in the literature [4] was used for the induction of CaOx crystallisation in whole urine. Pooled urines from both groups were warmed to 37°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 (NaOx) at pH 5.9–6.5. After 1 h, a further 15 ml/l of NaOx was added to the mixture. An hour later, the crystals were harvested by centrifugation at 8,000*g* for 30 min at room temperature.

Crystals were demineralised in 10.0 ml 250 mM EDTA, pH 8.00, per gram of crystals for 3 days with continuous stirring at 4°C. Preliminary experiments were performed to optimise the volume of EDTA required for maximising the yield of albumin. This was achieved by dissolving 1.14 g of crystals from the urines of white subjects in different volumes of EDTA and analysing the supernatant extracts by 10% SDS-PAGE and western blotting. The optimum volume was found to be 10 ml. After demineralisation, the supernatants BCME [crystal matrix extract (CME) from blacks] and WCME (CME from whites) were dialysed against distilled water for 24 h at 4°C, then lyophilised and resuspended in distilled water (100 μl of water per 10.0 mg of extract).

Immunoaffinity protein purification

Protein-G sepharose (0.4 g) was washed three times in 10 ml PBS, pH 7.50, transferred to 10 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 ml PBS. Anti-HA was crosslinked to the protein-G sepharose by resuspending the mixture in 20.0 mM dimethylpimelimidate (DMP), 3.0 M NaCl, 50.0 mM borate buffer, pH 9.00 and incubating for 1 h. The anti-HA-sepharose was washed with 200 mM ethanolamine, pH 8.00, resuspended in 200 mM ethanolamine, and incubated at room temperature for 2 h. Finally, the mixture was centrifuged for 1 min, washed with PBS and transferred to a 2 ml column.

Albumin samples derived from both ultra-filtered urine and CME were separately dissolved in water, applied to the anti-HA-sepharose column at a flow rate of 0.4 ml/min, and the column was then washed overnight with PBS to ensure the removal of any unbound material. Albumin was then eluted with 100 mM glycine, pH 2.80 and the eluant 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 Bio-Rad protein assay was used to determine the protein concentration of the dialysate. The purity of the isolated protein was analysed by 10% SDS-PAGE and western blotting.

Western blotting

Following SDS polyacrylamide gel electrophoresis, the proteins were transferred to a nitrocellulose membrane at 100 V using transfer buffer (150 mM glycine, 20% methanol 20.0 mM Tris, pH 8.30). The nitrocellulose membrane was incubated for an hour in blocking buffer (20.0 mM NaCl, 5% skim milk, 0.1% Tween-20 and 50.0 mM Tris, pH 7.40) and then immersed in anti-HA (Sigma) at a 1:1,000 dilution before extensive washing with blocking buffer. Incubation of the membrane with the secondary antibody (peroxidase-labelled affinity purified antibody, KPL Europe Guildford, UK) was carried out at a dilution of 1:2,000 and after further washing the blot was developed using 4-chloro-1-naphthol in methanol.

Crystallization experiments

Subjects and urine collections

About 24 h urines were collected in 2 l plastic bottles from eight male subjects in each race group. Samples which tested positive for blood or infection were discarded. Urines were analysed for their routine biochemical composition and were then filtered as previously described, in order to remove cellular debris. Relative supersaturation (RS) values of CaOx, brushite and uric acid were computed from urine composition data using the EQUIL program [15]. Samples were then ultra-filtered using an Amicon hollow fibre bundle (H1P 10) with a nominal cut-off at 10 kDa. Ultra-filtered urines from black and white subjects (BUF and WUF, respectively) were used for the determination of the CaOx metastable limit and [^{14}C]-oxalate crystal deposition kinetics as described below.

Calcium oxalate metastable limits (MSL)

The metastable limits of each UF sample was determined to ascertain the NaOx load required to initiate crystallization in the kinetics experiment. Briefly, each sample was dosed with a series of NaOx solutions of progressively increasing concentration (range 0.015–0.195 mol/dm^3 in steps of 0.015 mol/dm^3) until nucleation of CaOx crystals occurred, as detected by a sharp increase in turbidity ($OD_{620\ nm}$) measured with a UV spectrophotometer (Helios Gamma, Cambridge, UK). The concentration of NaOx corresponding to the sudden increase in $OD_{620\ nm}$ was taken as a measure of the CaOx MSL.

Albumin samples

Albumin was isolated from the urines of 20 black and 20 white subjects as described earlier (BA and WA, respectively). After establishing the respective purities of samples obtained from both UF and CME sources, they were combined for the crystallization studies. Human serum albumin was obtained from Sigma. Its purity was confirmed by SDS-PAGE and immunoblotting.

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

About 15.0 ml of BUF was transferred to each of four 100 ml glass flasks labelled "CTRL" (control), "HSA", "WA", and "BA". About 15.0 μg of the respective proteins were added to the latter three flasks to give final concentrations of 1.00 μg/ml. Since the volumes required for the delivery of 15 μg were 17.5 μl for HSA and WA, and 18.8 μl for BA, significant dilution of BUF did not occur. The same procedure was followed for WUF. The flasks were incubated in a shaking water bath (100 rpm) at 37°C for 15 min. About 7.50 μl of 3.125 μCi/100 ml [^{14}C]-oxalic acid (NEN, Boston, USA) was added, followed by 150 μl of "cold" NaOx at a concentration of 0.030 mol/dm^3 in excess of the previously determined metastable limit. This was taken as time $t = 0$. Thereafter, 2.50 ml of the UF-protein solution was aspirated from each flask and syringed through a filter into 0.25 ml concentrated HCl to quench the reaction. About 1 ml of this mixture was then transferred into 10.0 ml scintillation fluid (Zinsser Analytic, Frankfurt). This process was repeated at 30, 60, 90 and 120 min. A scintillation counter (Malvern Instruments, UK) was used to determine the amount of [^{14}C]-oxalate remaining in solution at these times. The experiment was performed in duplicate.

Preparation of CaOx monohydrate crystals for measurement of zeta potentials and inhibition of CaOx crystal aggregation

Calcium oxalate monohydrate crystals (COM) crystals were prepared according to the method of Pak et al. [16]. Briefly, 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 7 days at 6°C. Crystals were then filtered through a 0.22 μm filter paper, washed with 2.0 ml of methanol and dried in an oven at 95°C for 60 min. X-ray powder diffraction analysis confirmed that the crystals were 100% COM.

Zeta potential measurements

Monohydrate crystals crystal slurries were prepared by addition of COM crystals (0.3 mg/ml) (prepared as described above) to 10.0 mM sodium acetate buffer, pH 5.70. These were equilibrated overnight with constant stirring (1,100 rpm) at 25°C [11]. Aliquots (11.0 ml) were then transferred to each of four vials. One of these was set aside as the control while HSA, BA and WA were added separately to each of the other three vials to yield final concentration of 1.00 μg/ml for each of the respective proteins. The added volumes were sufficiently small to avoid dilution of the slurry. Stirring was continued for 2 h at 37°C and 1,100 rpm. The zeta potential (zp) of the protein—COM slurry in each vial was measured in duplicate using a Zetasizer 4 (Malvern Instruments, UK). The experiment was repeated five times.

Inhibition of COM crystal aggregation

The spectrophotometric assay developed by Hess and co-workers [9] was used for the measurement of the inhibition of COM crystal aggregation in the absence (control), and presence of HSA, BA and WA. Previously prepared COM crystals were dissolved in 10.0 mM Tris–HCl buffer, containing 90.0 mM NaCl, pH 7.50 to yield COM slurries of concentration 0.8 mg/ml. Aliquots (7.00 ml) of these slurries were added to each of four vials and were equilibrated overnight in a water jacket set at 37°C, with continuous stirring at 1,100 rpm. After equilibration, HSA, BA and WA were added separately to three vials to yield final concentrations of 1.00 μg/ml for each of the respective proteins. As before, dilution did not occur. Stirring was continued for 2 h at 37°C and 1,100 rpm. The fourth vial was set aside as a control.

An aliquot (2 ml) of the protein–COM slurry was then transferred to a 10.0 mm glass cuvette in a UV spectrophotometer (Helios, Cambridge, UK). The cell holder was thermostatted at 37°C and the contents were stirred continuously at 1,100 rpm. $OD_{620\ nm}$ was measured at intervals of 5 s until it remained constant. The stirrer was then switched off and $OD_{620\ nm}$ was recorded until the decline in absorbance due to particle settling had stopped [9]. The gradient of this part of the curve gives the rate of sedimentation, Ts, due to aggregation. The percentage inhibition of COM crystal aggregation is given by 100-100 Ts/Tc where Tc is the sedimentation rate of the control sample [9]. The experiment was performed five times.

Statistical analysis

Urine composition data, zeta potentials and aggregation inhibition values were subjected to ANOVA to determine significant differences.

Results

Protein purification

The binding efficiency of the anti-HA column was checked using commercial HSA and was found to be satisfactory. Yields of purified albumin from concentrated BU (0.613 mg/l) and WU (0.325 mg/l) as well from BCME (0.802 mg/l) and WCME (0.743 mg/l) were not significantly different. The proteins ran as single well-defined bands on 10% SDS-PAGE with molecular weights corresponding to the 60 kDa-HSA (Fig. 1a). The identity of the bands was further confirmed by immunoblotting (Fig. 1b). The protein was also purified using cyanogen bromide-coupled anti-HA-sepharose. However, the random orientation of the antibody with respect to its site of attachment resulted in inferior binding efficiency (data not shown). No differences were observed from SDS-PAGE and western blotting with respect to albumin prepared by urine concentration or from CME.

Crystallization experiments

Mean urinary concentrations and other physicochemical variables for the two groups are given in Table 1. It is noted that some variables are significantly different between the groups.

The effects of albumin on the deposition kinetics of labelled [^{14}C]-oxalate in BUF and WUF are shown in Figs. 2, 3, respectively. It is apparent that the initial deposition kinetics in both urines appears to be complete within 30 min of crystal initiation. Accordingly, deposition rates were calculated from the respective gradients during this initial time period. These revealed that the relative rates were WA < BA = HSA < CTRL in BUF and BA < WA < HSA = CTRL in WUF.

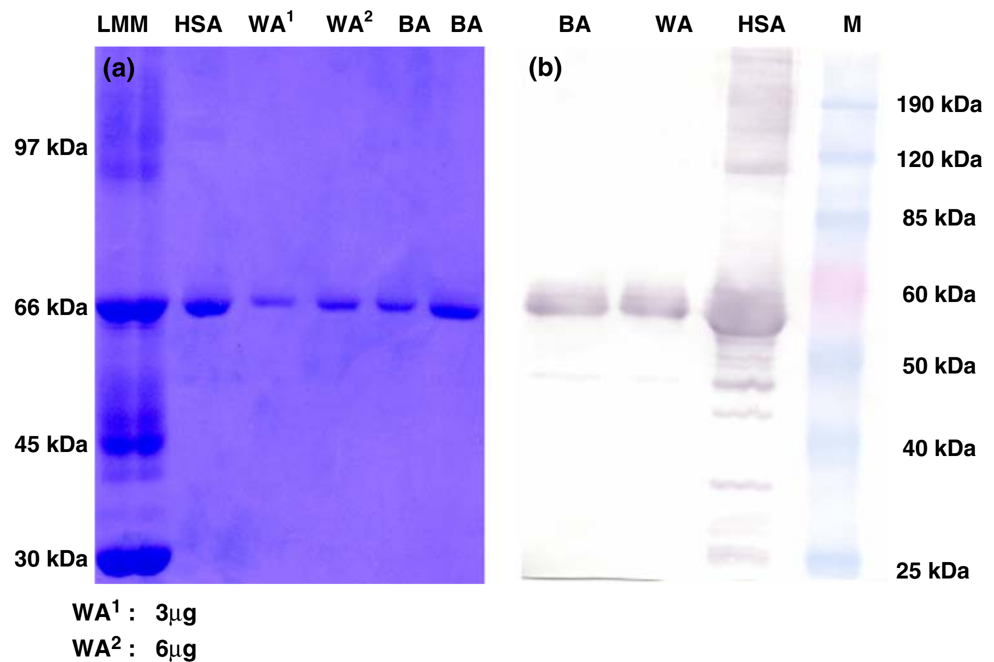

Fig. 1 Purification of urinary human albumin. Immunoaffinity purified human albumin isolated from black (BA) and white (WA) subjects: 10% SDS polyacrylamide gel (**a**) and western blot (**b**) with a rabbit anti-HA polyclonal antibody (Sigma). Bands in (**a**) were identified using Coomassie blue while in (**b**) the membrane was developed using 4-chloro-1-naphtol

The mean zeta potentials of COM crystal slurries in the presence of each albumin sample and the independently determined mean percentage aggregation inhibition are given in Table 2. It is noted that the magnitude of the negative zp follows the trend BA > WA > HSA > CTRL which mimics that for the percentage inhibition. It is also noted that BA is a significantly superior inhibitor of COM aggregation than HSA and WA.

Discussion

Previous in vitro studies of the effects of albumin on CaOx crystallization have involved testing a commercially available HSA in inorganic solutions. We are aware of only one study which employed urine-derived albumin [13] and only one which used real urine as the test medium [10]. We believe that the present study is the first in which urine-derived albumin has been tes-

Table 1 Mean urinary concentrations and physicochemical data of black (n = 8) and white subjects (n = 8) (and standard deviations)

	Black subjects	White subjects	P
Volume (ml/24 h)	1,508 (292)	1,316 (397)	0.46
pH	6.48 (0.46)	6.28 (0.34)	0.52
Sodium (mol/l)	0.1110 (0.1300)	0.0977 (0.0456)	0.85
Potassium (mol/l)	0.0359 (0.0300)	0.0194 (0.0173)	0.37
Calcium (mol/l)	0.0033 (0.0015)	0.0045 (0.0017)	0.32
Magnesium (mol/l)	0.0024 (0.0005)	0.0025 (0.0005)	0.74
Phosphate (mol/l)	0.0154 (0.00026)	0.0247 (0.0065)	0.03*
Oxalate (mol/l)	7.39×10^{-5} (1.96×10^{-5})	9.82×10^{-5}(2.12×10^{-5})	0.13
Citrate (mol/l)	0.0019 (0.0010)	0.0021 (0.0002)	0.66
Uric acid (mol/l)	0.0021 (0.0003)	0.0030 (0.0008)	0.07
Chloride (mol/l)	0.1340 (0.0981)	0.1160 (0.0338)	0.74
RS brushite	1.89 (1.65)	2.61 (1.39)	0.53
RS calcium oxalate	2.45 (1.61)	3.80 (2.09)	0.31
RS uric acid	0.84 (0.58)	1.64 (0.52)	0.34
MSL calcium oxalate (mol/l)	0.105 (0.010)	0.045 (0.010)	0.01*

RS relative supersaturation [15] *MSL* metastable limit

*Statistically significant at $P < 0.05$

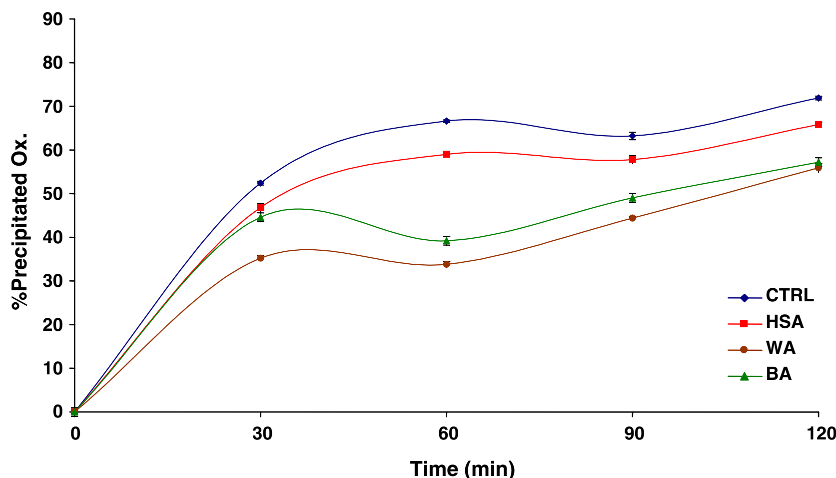

Fig. 2 Crystal deposition kinetics in BUF using labelled [^{14}C]-oxalate

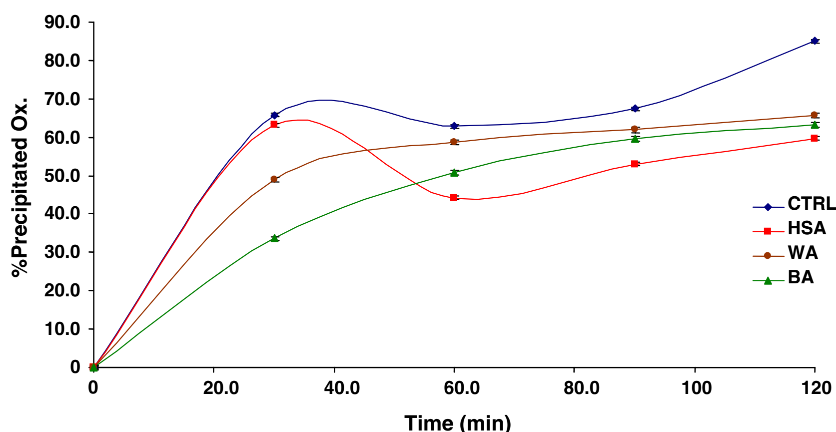

Fig. 3 Crystal deposition kinetics in WUF using labelled [^{14}C]-oxalate (*error bars* are present but are very small)

ted in real urine and that, furthermore, it is the first in which the protein has been studied in the urine from which it was isolated. As such, our findings in the [^{14}C]-oxalate deposition experiments are of some importance. These show that urinary albumin, irrespective of its ethnic origin and irrespective of the urine in which it is tested, is an inhibitor of the rate of CaOx crystal

Table 2 Mean zeta potentials (and standard deviations) and mean percentage inhibition of COM aggregation (and standard deviations)

	CTRL[1]	HSA[2]	BA[3]	WA[4]
Zeta potential (mv)	–13.14 (0.51)	–14.36 (1.69)	–19.32 (3.20)	–16.90 (3.37)
Inhibition of aggregation (%)		30.62 (4.17)	50.68 (1.31)	35.02 (8.43)

Pairwise comparison of mean zeta potentials1 versus 2: P = 0.160, 1 versus 3: P = 0.003, 1 versus 4: P = 0.039, 2 versus 3: P = 0.016, 2 versus 4: P = 0.171, 3 versus 4: P = 0.273

Pairwise comparison of mean percentage inhibition

2 versus 3: P < 0.001, 2 versus 4: P = 0.33, 3 versus 4: P = 0.003

formation (Figs. 2 and 3). This contradicts the results of previous studies, which found it to be a promoter of nucleation [13, 14]. However, the latter studies employed different crystallization assays to those used in the present investigation; clearly, comparisons are more meaningful when the same techniques are used.

Our results also show that the origin of the urine in which inhibition is measured has an effect on albumin's performance. Worcester [17] has previously made the point that the activity of inhibitory proteins is determined by solution characteristics while we have reported a synergistic relationship between the inhibitory activity of other proteins and urine composition [1, 2]. In the present study, the absolute and relative inhibition by BA, WA and HSA is different in BUF and WUF, possibly due to subtle compositional differences noted in the urines themselves (Table 1). These differences need not necessarily be statistically significant. Indeed, while only one urinary component (phosphate) was found to be significantly different in these two cohorts, each urine will nevertheless have provided a different chemical environment, the synergistic com-

position of which could possibly have influenced the inhibitory activity of the albumin samples. It should not therefore be surprising that any particular albumin sample in the present study would behave differently in BUF and WUF. However, the observation that the three samples themselves are different suggests the possible existence of molecular differences between them.

Further evidence supporting the notion that subtle differences in the urinary composition of BUF and WUF could cause substantial differences in their crystallization potentials is provided by the metastable limit values. These show that the MSL in BUF is significantly greater than in WUF (Table 1), thereby demonstrating that initiation of CaOx crystallization occurs with greater difficulty in the former than in the latter. This is entirely consistent with epidemiological observations of a much lower incidence rate of CaOx urolithiasis in the black population and agrees with results, which we have reported previously [18].

Our zeta potential and sedimentation data provide further support for our hypothesis that molecular differences exist in the albumin samples. Hess and co-workers [7] have pointed out that since albumin binds to CaOx crystal surfaces, it is expected to affect surface properties and hence crystal aggregation. One such surface property is zp. Our zp values demonstrate that COM crystals coated with BA carry a negative charge of greater magnitude than those coated with WA and HSA (Table 2). This implies that the three albumin samples have different adsorption potentials or binding affinities for COM crystals, which itself is suggestive of differences in molecular structure between them. Independent of this point, however, is the implication of the different zp values themselves. Since COM crystals coated with BA have zp values of greater magnitude than those coated with WA and HSA, they will experience stronger inter-crystal repulsive forces and are thus less likely to aggregate. This is confirmed by our sedimentation experiments in which the inhibition of COM crystal aggregation was determined and was found to mimic the trend shown by the zp measurements (Table 2). The results of these two independent experiments (zp and sedimentation velocity) have thus yielded the same results, namely that albumin, irrespective of its origin, is an inhibitor of COM crystal aggregation and more importantly, that BA is superior to WA and HSA in this context. Since zp is not the only property which determines aggregation (or inhibition thereof), the possibility of differences between the three albumin samples at the molecular level, again arises.

Our hypothesis that differences might exist in the molecular structure of albumin extracted from different sources is reasonable when considered in the light of the numerous possible conformations described by Peters and Reed [19]. They have drawn attention to pH dependent isomerization being a cause of heterogeneity in terms of its influence on the loop and domain structures of the albumin molecule. A conformation-function relationship is therefore feasible.

Our observation of different behaviour by urinary and serum albumin has been reported by other workers who found that the former has a stronger promotory activity towards CaOx nucleation [13]. Although they do not believe that primary structural differences could be the explanation, they suggest that secondary modifications such as glycosylation might be considered [13]. This hypothesis can be extrapolated to urinary albumin from black and white South African subjects and should be explored in future studies. Another possible explanation for the different inhibitory properties of BA, WA and HSA is that different degrees of polymerization might exist in the three albumin samples in the present study. Cerini and co-workers have pointed out that albumin's capacity to form aggregates is well known and that aggregation of proteins generally leads to enhanced activity [13]. Since these workers observed a greater abundance of urinary albumin aggregates in healthy subjects than in idiopathic CaOx stone formers, we speculate that a similar relative difference might exist between subjects from the stone-free black population group and subjects from the stone-prone white group in South Africa. This hypothesis should also be investigated in future studies.

The emergence in this study of albumin from black subjects as being a superior inhibitor (of CaOx aggregation) to that from white subjects compliments our previous studies on urinary prothrombin fragment 1 [1] and Tamm Horsfall glycoprotein [2] in which the respective inhibitory capacities in black subjects were also found to be superior. Nevertheless, despite the interesting results in the present study, we recognise that CME was extracted from only two healthy black subjects. Further studies involving a greater number of participants are necessary before definitive differences between the groups can be confirmed. The results of the present study also provide motivation for future studies in which differences between urinary albumin from healthy subjects and stone formers should be investigated, possibly by application of a proteomic approach involving 2D gel electrophoresis and mass spectrometry.

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