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Urological Research

Hydration feature of urinary compounds

Evidence for molecular abnormality in calcium oxalate urolithiasis

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Summary. Proton-relaxation-time measurements were performed on lyophilized urine samples collected from 11 recurrent calcium oxalate stone-formers, 9 uric acid stone-formers, 9 patients with various urological disorders, and 20 normal individuals. The T_1 and T_2 relaxation times were determined using a Bruker PC Multispec at 20 MHz at 37°C for measurements of lyophilized sample and thereafter during gradual controlled rehydration. The prolongation of the relaxation times as a function of rehydration was found to differ significantly ($P<0.005$) between, on the one hand, the calcium oxalate stone-formers and, on the other hand the normal, uric-acid stone-formers, and patients with other urological disorders. Water compartmentalization was then calculated according to the fast proton diffusion model. At most of the experimental points during rehydration process, significantly ($P<0.001$) less water was bound to the compounds of urine from calcium oxalate stone-formers than that obtained from normal individuals. The variations in the bound hydration water may have been associated with possible changes in the structure or configuration of the compounds present in the urine of the different groups. The results reflect differences in the urinary content and/or properties of normal subjects and calcium oxalate stone-formers, and indicate that the mechanism of bound water relaxation is in some way specific to the pathophysiological state of urine.

Key words: Urinary macromolecules – Rehydration – Kidney stones – Urinary inhibitors – Nuclear magnetic resonance

The kinetics and mechanisms of renal stone formation are complex phenomena which have not yet been completely elucidated. Stone formation can not be attributed to any single factor [1], and it is now generally accepted as being due to a disturbance in the balance between the supersaturation of urine with a given salt and the concentration of various inhibitors and promoters of crystallization [2, 3]. Stone formation is an indication that the balance between supersaturation and inhibition has been disturbed in favor of the formation of large particles consisting of large individual crystals and/or large agglomerates of crystals [4–6].

Most renal calculi contain calcium, frequently in the form of composites of various species of calcium oxalate (CaOx) and calcium phosphate [7]. Several in vitro studies have indicated that a variety of inorganic and organic urinary compounds are effective as crystallization inhibitors [8–16]. Compounds such as chondritin sulfate, phosphocitric acid, glycosaminoglycans, acidic amino acids, and macromolecules containing detectable residues of gammacarboxyglutamic acid are believed to fulfil the physiological role of inhibitors in the urine of normal individuals. Most of the inhibition observed in normal human urine is said to be due to the presence of macromolecular species and not to trace amounts of low-molecular-weight compounds [13]. Inhibitors may affect the crystallization processes at minute concentration, since they need to interact with only relatively few energetically active growth sites on the crystal surface [4–6]. Therefore, it is extremely difficult to detect and characterize these minute amounts [13, 14]. Recently, we reported on the use of a proton magnetic resonance (PMR) technique to characterize the association of urinary compounds with water [17], and found that there are more hydrophilic compounds in the urine of normal (N) individuals than of stone-forming (SF) patients.

The compounds in the urine of SFs seem to differ from those of Ns with regard to the amount of water-binding sites and the water multilayer thickness surrounding them. These differences in the association of urinary compounds with water most probably maintain the structure and conformational integrity of these compounds and affect their biological activity. Because of the important physiological role played by these compounds, we expanded our study and now report the further characterization of urinary compounds obtained from the whole urine of patients with various urinary disorders, in order to describe their hydration properties.

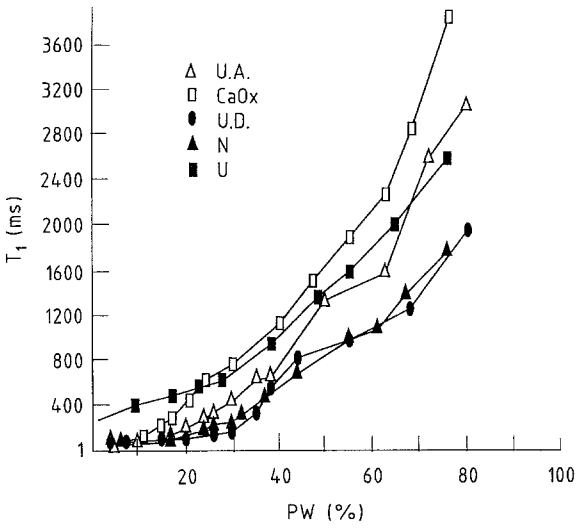

Fig. 1. The means of T_1 relaxation times of lyophilized urine samples from a urine-like solution (U), calcium oxalate stone formers (CaOxSF), uric acid stone-formers (UASF), patients with various urological disorders (UD), and normal (N) individuals as a function of the percentage added water (PW)

Patients and methods

Patients and normal subjects

Freshly voided urine was collected from 11 patients (1 female) with recurrent CaOx nephrolithiasis (CaOxSF) aged between 29 and 56 years (mean, 44 years), and 7 patients with uric acid nephrolithiasis (UASF) aged between 39 and 60 years (mean, 50 years). The controls were divided into two groups. The first group consisted of 9 (3 women) patients with other urinary disorders (not related to stone formation, UD) aged 19–66 years (mean, 45 years). Of these patients, 3 had presented with renal colic but no stone had been found, while 3 had genitourinary trauma (2 with kidney contusion and 1 with ureteral rupture), 1 had varicocele, 1 had an entero vesical fistula, and 1 was suffering from stress incontinence. The other control group consisted of 20 normal individuals (9 women; N), aged between 22 and 49 years (mean, 36 years).

Urine was collected over a period of time during which each patient was allowed a free choice of diet, and each urine sample was processed separately. All urine samples were lyophilized simultaneously.

Sample preparation

The osmolarity and uric-acid and calcium-ion concentrations of all urine and blood samples were measured. The urine was then placed in small vials and freeze-dried under controlled mild temperature conditions in order to avoid possible damage to the urinary compounds. The final temperature of the lyophilized material was approximately 10–12°C. The vials containing the lyophilized material were sealed under vacuum and stored at 4°C.

Magnetic resonance (MR) measurements

Determinations of relaxation time (T_1 and T_2) were performed on 250-mg lyophilized urine samples using a Bruke PC-20 Multispec, operating at 20 MHz and 37 ± 1°C. The T_1 relaxation time was measured by the 180°–τ–90° (inversion recovery) pulse sequence. The T_2 relaxation time was measured using the Carr-Purcell-Meiboom-Gill (CPMG) multi-echo pulse sequence. The T_2 value was determined by a linear fit of the logarithmic value of 20 echo amplitudes as a function of decay time. All signals were recorded using phase-sensitive detection. The relaxation times were measured first on dry samples and then during gradual rehydration up to 80% water content (% PW). The samples were measured nine times to increase the signal-to-noise ratio, and each experimental point was repeated at least twice. The size of the bound water fraction (FB) was calculated from the T_1 and PW values, as suggested by the fast proton diffusion (FPD) model [18, 19]

$$FB = \frac{R1 - Ra}{Rb - Ra}$$

where R1 = the measured relaxation rate ($1/T_1\ s^{-1}$); Ra = relaxation rate for bulk water ($0.37\ s^{-1}$); and Rb = relaxation rate for hydration water ($16.2\ s^{-1}$ at 20 MHz).

Urine-like solution

Fresh urine-like solution was prepared as described previously [16] and then processed in the same way as the urine samples.

Statistical analysis

Mean, standard error of the mean, regressing lines best fitted by the least squares method, correlation coefficient of the regression, and analysis of variance were used to assess statistical significance; $P < 0.05$ was considered significant.

Results

Rehydration profiles

The consecutive measurements of the relaxation time (T_1, T_2) of lyophilized urine being gradually rehydrated as a function of PW are defined as rehydration profiles. The profiles (mean of the relaxation time's value ± SD) for N, CaOxSF, UASF, and UD are shown in Fig. 1 and 2 for T_1 and T_2 relaxation times vs. PW, respectively. Significantly ($P < 0.005$) prolonged T_1 and T_2 values were measured during gradual rehydration in all groups. However, the pattern of the prolongation of the relaxation times was different for the various groups. The T_1 values for the lyophilized urine samples of the CaOxSF group increased rapidly after the addition of less water (PW = 6%) than that required to produce similar effects in the urine of UASF (PW = 12%) patients or the N and UD groups (PW = 20%). The rehydration patterns were, empirically best represented by the regression of a second-order polynomial. The T_2 relaxation times of lyophilized urine (Fig. 2) from all groups were prolonged through out the rehydration processes, but the T_2 values of the CaOxSF and UASF groups increased more rapidly than those of the other groups.

Urine analysis

The osmolarity value of blood and urine of each individual was determined using an Osmett A automatic osmometer (Precision Systems, Natic, Massachusetts, USA).

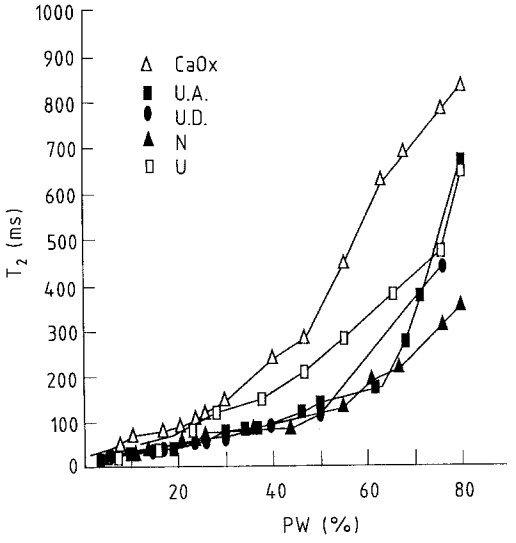

Fig. 2. The means of T_2 relaxation times of lyophilized urine samples from a urine-like solution (U), calcium oxalate stone formers (CaOxSF), uric-acid stone-formers (UASF), patients with various urological disorders (UD), and normal (N) individuals as a function of the percent age of added water (PW)

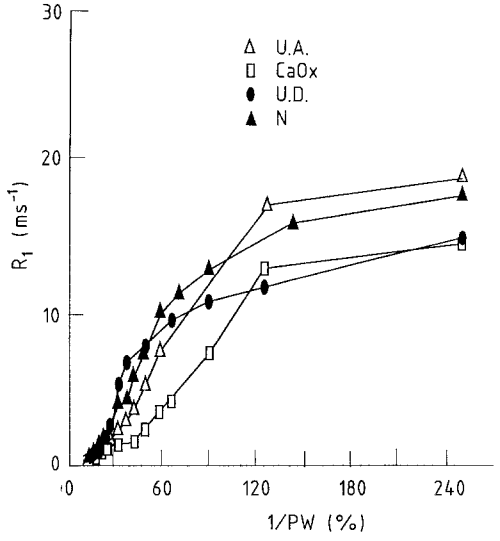

Fig. 3. The longitudinal relaxation rate (logR1) of lyophilized urine samples from calcium oxalate stone formers (CaOxSF), uric acid stone-formers (UASF), patients with various urological disorder (UD), and normal (N) individuals as a function of the reciprocal value of the percent age of water content (1/PW)

Table 1. The mean ± standard error of osmolarity values in the blood and urine samples of calcium oxalate stone-formers (CaOxSF), uric acid stone-formers (UA), patients with other urological disorders (UD) and normal controls (N)

	Osmolarity values (mOsm)			
	CaOxSF	UA	UD	N
Blood	294 ± 3.1	293 ± 4.0	289 ± 2.6	288 ± 5.7
Urine	714 ± 236	530 ± 215	551 ± 252	872 ± 222

Water compartmentalization

The FB reflects the amount of water associated with the lyophilized material with respect to the total water content. The mean changes in the calculated FB during the rehydration processes are shown in Fig. 3, at most of the points during rehydration, significantly ($P<0.001$) less water was bound to the urine compounds of CaOxSF patients than of Ns. The FB of urines of the other groups was within the range determined for Ns and CaOxSF patients.

Discussion

The present results fully confirm our previous findings [17] that compounds from the urine of recurrent CaOxSF patients differ significantly from those of normal controls with respect to their association with water. In the present study, possible differences in hydrophobicity between the studied groups did emerge and it seems that the urine of normal individuals is characterized by elevated activity or levels of hydrophilic macromolecules as compared with that of SF patients.

Many studies [20–27] using PMR which have been carried out to explore the state of water in biological systems, have shown that this water exists in a more structured state than bulk water. It has been found that water in biological system exists in an adsorbed or structured state surrounding macromolecules [27]. The achial amount of structured water relative to total water, i.e., the FB in biological systems, is a subject of controversy [20–27]. In the present study, the amount of FB was not determined in whole urine, because the later is mainly composed of water and therefore the FB is so low as to be negligible; however, the water-binding profile of lyophilized urinary compounds was assessed (Fig. 3). This assessment revealed that compounds obtained from the urine of N individuals and patients with various urinary disorders have a higher water-binding capacity than those in the urine of CaOxSF patients. The different FBs, i.e., the tightly bound water, were primarily responsible for the different T_1 values observed in the rehydration experiments. However, the urine also contains free water associated in the coordination shells of ions, and proton exchange probably occurs among the various water fractions [28, 29]; therefore, T_1 would also be affected by all the water fractions in the urine.

The exposure of macromolecules to a concentrated salt solution can cause changes in macromolecular conformation and decreases in the fraction of water associated with the macromolecules [30, 31]. These changes alter the biological role of urinary macromolecules. Figure 1 shows the rehydration profile of a urine-like solution after its being lyophilized in the same way urine. The urine-like solution contained salts but no proteins or other macromolecules. Figure 1 reveals that the prolongation of T_1 was not affected by the amount of salts in the urine-like solution. Table 1 shows that the osmolarity values of the urine obtained from the CaOxSF and N groups were very

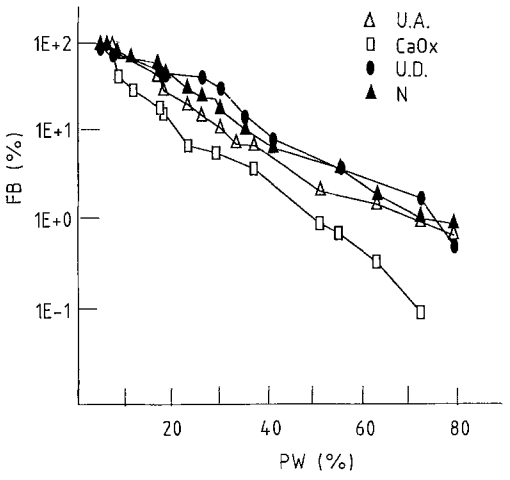

Fig. 4. The mean changes of the calculated fraction (%) of bound water (FB) in rehydrated lyophilized urine from calcium oxalate stone-formers (CaOxSF), uric acid stone-formers (UASF), patients with various urological disorders (UD), and normal (N) individuals as a function of the water content (PW)

similar. Therefore, it seems that the significant differences in the rehydration and FB profiles of these groups were most probably due to differences in the amount or activity of their macromolecules. A high T_1 value for urine indicates more free water in the sample. Any changes in the relative amount of free and bound water molecules (the FB) would change the T_1 value. Thus, a change in T_1 may be the result of: (a) alterations in the amount of bound water in the rehydrated urine sample [23]; (b) differences in the composition or amount of "structure breaking" or "structure making" ions (i.e., K, Na, etc.); or (c) changes in the volume of macromolecular coordination shells (e.g., as a result of alterations in macromolecules conformation). In addition, these effects are not always independent, since the first two possitiolities may lead to change in protein conformation, thus altering the coordination of shell volumes [24–31]. The pronounced and rapid prolongation of T_1 and T_2 profiles during rehydration processes (Fig. 1 and 2) could be explained on the basis of more free water being present in the urine volume, as can be seen in Fig. 3.

It is reasonable to assume that water on the surface of or near macromolecules may activate functional groups and initiate biological activity. The rehydration process is likely to activate chemically the functional groups of macromolecules in the lyophilized urine. During the late stages of rehydration, the T_1 relaxation times of all groups approached the T_1 value of fresh urine and urine-like solution (2.5–3 s). On the other hand, before adding water to the lyophilized urines and during the first stages of the rehydration process, the T_1 values of the various groups differed from each other. Figure 3 shows the linear relation of the T_1 relaxation rate ($R1 = 1/T_1\,s^{-1}$) and the reciprocal of PW. In all groups, this relationship could be described mathematically by a linear section and a constant region. According to the FPD model [18, 19], the point of singularity – determined by the intersection of the best-fit lines to the data in individuals sections – defines the hydration fraction (HF) of the materials in the tested urine (the hydration fraction is the amount of bound water per unit mass of solids). Therefore, the differences in the slopes of the curves of the various groups are related to the different HFs of the samples. The calculated HFs for the different groups studied were 16% for NS, 7% for CaOxSF patients 10% for UASF patients and 18% for UD patients. The results obtained from the rehydration experiments of the lyophilized urine-like solution and the good agreement of Fig. 3 with the theoretical description of water binding by macromolecules [21] justify neglecting the inorgenic contribution to the change in the relaxation rate (R_1). In the rehydration experiment, first, all the bound water sites were filled, and the excess of water could then be considered as bulk water. Therefore, the HF value represents the point in the rehydration process at which the surface of the macromolecules is effectively saturated with water. The variations in the relative amount of bound hydration water may be associated with possible changes in the structure or configuration of the macromolecules. Water hydrogen binds with charged or polar sites to macromolecules, while interactions with non polar sites are known to cause changes in water-water binding, this being referred to as "hydrophobic binding" [23, 27]. These interactions cause most of the charged and strongly polar sites to be on the surface of the folded macromolecules, while apolar sites are preferentially buried inside the folded molecule. Consequently, the hydration process contributes greatly to the determination of shape-specific properties of the urinary macromolecules and is crucial to initiating and maintaining their biological activity as specific inhibitors of calcium kidney stones. These results strongly confirm those reported by Nakagawa et al. [13].

In conclusion, the present results indicate that the mechanism of bound water relaxation is in some way specific to the pathophysiological state of urine, and they point to the possibility of detailed urine discrimination using, MR parameters. It is still not known whether this may have a clinical application for following up patients and predicting treatment efficacy. It is hoped that studies now in progress will help to resolve this question.

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