Urol Res (1992) 20:393–397

Urological Research

Effects of bacteria involved with the pathogenesis of infection-induced urolithiasis on the urokinase and sialidase (Neuraminidase) activity

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Accepted: 3 June 1992

Summary. It has been hypothesized that urinary urokinase and sialidase may play a role in urolithiasis. If these theories have substance it is to be expected that microorganisms may also affect these enzymes, since the association between urinary tract infection and renal stone formation is well known. It is generally assumed that *Proteus mirabilis* and *Staphylococcus albus,* which produce the urea-splitting enzyme urease, are responsible for stone formation. However, the importance of non-urease-producing microorganisms (*Escherichia coli* and *Enterococcus*) in urolithiasis is unclear. Spectrophotometric studies were therefore devised to clarify this problem. Microorganisms associated with infection-induced stones (*Proteus mirabilis* and *Escherichia coli*) respectively inhibited the urokinase and stimulated the sialidase activity. In contrast, microorganisms which were not associated with infection stones (*Bacillus subtilis*) had significantly less effect on urokinase and sialidase activity. This study may explain infection-induced stone formation and could open a completely new line of research.

Key words: Bacteria – Infection – Sialidase (Neuraminidase) – Urine – Urokinase – Uroliths

Although many preurinary, urinary, and chemical risk factors have been studied in connection with urolithiasis, hardly any research has been done on urinary enzymes such as urokinase (UK) and sialidase (SA). The activities of these enzymes differ significantly in the presence of calcium (promoter) and magnesium (inhibitor) ions [10], as well as in the presence of urine from persons with and without urolithiasis [7–9]. The average inhibition of UK activity by urine from persons with and without renal stones has been found to be $77.06\% \pm 12.72$ and $47.12\% \pm 11.72$ respectively ($P < 0.001$). In contrast to the inhibition of UK activity, SA activity increased with age in both types of urine. However, the SA activity of renal stone subjects was significantly higher than that of healthy counterparts ($P < 0.001$). These results support the hypothesis that SA may be responsible for the conversion of urinary mucosubstances to mineralizable matrix and that UK regulates the urinary uromucoid concentration. If these theories have substance, it can be anticipated that microorganisms may affect these enzymes too, since the association between urinary tract infection and renal stone formation is well known [1, 3]. It is generally theorized that *Proteus mirabilis* and *Staphylococcus albus,* but not *Escherichia coli,* are associated with struvite and carbonate apatite uroliths [1]. An essential requirement for these types of uroliths is the presence of microorganisms that produce the urea-splitting enzyme urease. Urealysis results in increased excretion of ammonium ions and carbonate apatite in urine. According to the urease theory, supersaturation of these compounds is required for crystallization to occur. The importance of non-urease-producing microorganisms (*E. coli, Enterococcus,* etc.) in urolithiasis is unknown, and no role for them has been postulated. Microbial studies were therefore devised to test the hypothesis that bacteria associated with uroliths would respectively inhibit and stimulate UK and SA activity, which would result in more favorable conditions for urolithiasis. The most potent infection is expected to be the one that has an effect on both UK and SA activity.

Materials and methods

Reagents and chemicals

All reagents were of "Analar" grade. Boehringer (Mannheim, FRG) supplied NADH, rabbit muscle lactate dehydrogenase in ammonium sulfate solution, *N*-acetylneuraminic acid aldolase from *E. coli,* SA (neuraminidase) from *Clostridium perfringens,* and bovine colostrum *N*-acetylneuraminosyl-D-lactose (sialyllactose). The reagents sodium phosphate, EDTA and Triton X-100 were obtained from Merck (Darmstadt, FRG) and BDH (Poole, Dorset, England). Sigma Chemical Co. (St. Louis, Mo., USA) supplied the substrates plasminogen (human plasma) and D-valyl-L-leucyl-L-lysine *p*-nitroanilide, as well as UK from human kidney. Nutrient broth no. 2 was produced by Oxoid (Basingstoke, Hants., England).

Microorganisms

Nutrient broth medium (×0.1, diluted with water) was used as growth medium for all the cells. Inoculated flasks were incubated overnight under a nitrogen gas phase at 37°C, centrifuged (1700 *g*, 30 min, 5°C), suspended in nutrient broth, and counted on a hemocytometer (Neubauer).

Urokinase determination

The activity of the UK/plasmin system was assayed according to a modified method of Wiman et al. [12]. Briefly, 400 µl activator reagent was added to 200 µl nutrient broth (×0.1, diluted with distilled water) and 228 µl 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 10 mmol/l EDTA and 0.1 g/l Triton X-100. The activator reagent was composed of 1.0 µmol/l plasminogen and 0.6 mmol/l D-valyl-L-leucyl-L-lysine *p*-nitroanilide, dissolved in 0.1 mol/l sodium phosphate buffer. All the additions were performed in ice. The reaction was initiated by the addition of UK (EC 3.4.21.31). The total volume was 828 µl. After 2 min at 37°C, the enzyme activity was estimated from the product concentration recorded at 405 nm on a Hitachi spectrophotometer 150-20 (Tokyo, Japan) attached to a data processor. The molar extinction coefficient for *p*-nitrophenol was taken as 9620 $mol/l^{-1}cm^{-1}$ [11]. The blank consisted of buffer, nutrient broth and activator reagent.

To determine the effect of microorganisms on the UK/plasmin system, the same method was followed as described except that this assay was performed in a stirred water bath for 90 min at 37°C under a nitrogen gas phase. Incubation times in the time-study experiments obviously varied. This determination consisted of (1) a blank and a control, and (2) a blank plus bacterial cells and a control plus bacterial cells. The same amount of UK was added to all the controls (av. 0.2 IU). The total volume for each was 828 µl. After the desired incubation time, tubes were placed in ice before the absorbance was read at 405 nm. The blank rate value was subtracted from the rate value obtained in the presence of the enzyme to obtain the real control value. This procedure was repeated with the blanks and controls containing bacteria, which allowed the effects of cells on the synthetic UK substrate to be eliminated. The UK experiments were each repeated four times in duplicate.

Sialidase determination

The working suggestion for the spectrophotometric determination of the SA activity was supplied by Boehringer (Mannheim, FRG) [9]. Briefly, the reaction mixture of the blank consisted of 1650 µl Tris-buffer (50 mmol/l, pH 7.5), 260 µl nutrient broth (×0.1, diluted with distilled water), 20 µl NANA-aldolase (0.4 U), 10 µl freshly prepared lactate dehydrogenase (0.2 ml LDH in 0.8 ml distilled water), 30 µl NADH (10 mmol/l), and 200 µl sialyllactose (0.15 mmol/l), the substrate for SA. The total reaction volume of the ice-cold mixture was 2170 µl. The control reaction mixture was the same, except for the addition of 0.6 mU SA. The decrease in absorbance of the measurable variable NADH was measured at 334 nm in a Hitachi spectrophotometer 150-20 (Tokyo, Japan) at 37°C, which was connected to a data processor. The molar extinction coefficient for NADH was taken as 6220 $mol/l^{-1}cm^{-1}$ [6].

Sialidase activity in the presence of microorganisms was determined according to the described method except that the assays were performed in a stirred water bath for 90 min at 37°C under a nitrogen gas phase. SA time studies were also performed, as described in the section on UK determination. In these determinations there were once again blanks with controls, and blanks plus cells with controls plus cells. The total volume for each was 2170 µl. After the desired incubation time, tubes were placed in ice before the absorbance was read at 334 nm. The blank rate value was subtracted from the rate value obtained in the presence of SA to obtain the real control value. This procedure was also repeated with the blanks and controls containing microorganisms to eliminate additional microbial effects on the assay. For example, it is known that some bacteria, fungi and protozoans contain SA which, when not taken into consideration, may lead to erroneous conclusions [4, 5]. The SA experiments were each repeated four times in duplicate.

Statistical analysis

A two-way analysis of variance was applied to test for differences between microorganisms and number of cells or times with respect to the enzyme activity. Pairwise comparisons were done using the method of least square means.

A regression analysis was done to test whether there was a linear trend of enzyme activity as the number of microorganisms increased or as time elapsed. It was also tested whether the trend, if it existed, was different in the various microorganisms.

To simplify interpretation, all the observations were expressed as a percentage of the corresponding value at time zero or when no microorganisms were added.

Results

The effect of various bacteria on UK and SA activity is summarized in Tables 1 and 2. Increasing numbers of *P. mirabilis, E. coli* and *B. subtilis* cells inhibited UK activity (Fig. 1A) and stimulated SA activity (Fig. 1B). The slopes of the activities of UK and SA in the presence of different numbers of *P. mirabilis* and *E. coli* were significantly different from that of *B. subtilis* (Table 3). To investigate whether the UK inhibitor and SA stimulator were synthesized during incubation, a time study was devised. Although the inoculum sizes were the same in the time study (UK, 46×10^6 cells/ml; SA, 8×10^6 cells/ml) increased UK inhibition (Fig. 2A) and SA stimulation (Fig. 2B) were obtained, especially with *P. mirabilis* and *E. coli.* The slopes over time for the UK and SA activities of *P. mirabilis* and *E. coli* were significantly different from that of *B. subtilis* (Table 3).

The UK and SA activities in the presence of bacteria which produce urease either usually (+), occasionally (+, −) or never (−) are summarized in Table 4. Dominant bacteria in patients with infection-induced stones are also shown in Table 4.

Discussion

The present study showed that bacteria affected the activities of UK and SA significantly. According to the time studies, it seems highly probable that these inhibitors or stimulators were synthesized by the bacteria during incubation. The only questionable result was obtained with *P. mirabilis* regarding the significance of the slope of the SA activity (Table 3). In this case the values $r = 0.1257$ and $P = 0.5975$ were obtained after 150 min of incubation. However, these change to $r = 0.7952$ and $P < 0.0002$ when the incubation time is 90 min, which would result in a significant difference between the slopes for *P. mirabilis* and *B. subtilis.* A possible explanation for this phenomenon is that the *Proteus* cells started metabolizing the

Table 1. Mean urokinase activities (%) of *Proteus mirabilis* (PM), *Escherichia coli* (EC), and *Bacillus subtilis* (BS) in various quantities and after various time intervals

	Microorganisms			*P*-value for pairwise comparison		
	PM	EC	BS	PM vs EC	PM vs BS	EC vs BS
Time (min)						
0	100.00	100.00	100.00	1.0000	1.0000	1.0000
30	83.00	78.00	101.75	0.2556	0.0001	0.0001
60	68.25	69.75	92.50	0.7312	0.0001	0.0001
90	54.00	56.00	84.75	0.6471	0.0001	0.0001
150	49.25	34.25	81.50	0.0013	0.0001	0.0001
Cells ($\times 10^{-6}$)						
0	100.00	100.00	100.00	1.0000	1.0000	1.0000
8	90.00	72.00	90.25	0.0001	0.9519	0.0001
23	79.50	64.75	85.00	0.0007	0.1873	0.0001
46	68.00	46.75	79.25	0.0001	0.0083	0.0001
77	57.75	25.00	66.00	0.0001	0.0500	0.0001
116	40.25	8.50	66.75	0.0001	0.0001	0.0001
155	32.50	10.50	59.75	0.0001	0.0001	0.0001

Table 2. Mean sialidase activities (%) of PM, EC, and BS in various quantities and after various time intervals

	Microorganism			*P*-value for pairwise comparison		
	PM	EC	BS	PM vs EC	PM vs BS	EC vs BS
Time (min)						
0	100.00	100.00	100.00	1.0000	1.0000	1.0000
30	122.00	104.75	101.25	0.0001	0.0001	0.3561
60	122.75	110.25	106.50	0.0018	0.0001	0.3231
90	132.25	116.75	108.00	0.0002	0.0001	0.0245
150	107.00	119.50	103.25	0.0018	0.3231	0.0001
Cells $\times 10^{-6}$						
0	100.00	100.00	100.00	1.0000	1.0000	1.0000
4	135.00	117.25	107.00	0.0001	0.0001	0.0009
8	161.75	137.00	111.00	0.0001	0.0001	0.0001
16	173.25	161.75	109.75	0.0002	0.0001	0.0001
23	185.00	188.75	112.25	0.2023	0.0001	0.0001
46	187.75	181.00	118.00	0.0241	0.0001	0.0001

Table 3. Correlation *(r)* and regression *(b)* coefficients for urokinase (UK) and sialidase (SA) activity of PM, EC, and BS with respect to number of cells and time

Enzyme	Micro-organism	Number of cells			Time		
		b	*r*	*P*	*b*	*r*	*P*
UK	PM	−0.4226*	0.9453	<0.0001	−0.3427*	0.8674	<0.0001
	EC	−0.5478*	0.9190	<0.0001	−0.4208*	0.9628	<0.0001
	BS	−0.2364	0.9187	<0.0001	−0.1455	0.8391	<0.0001
SA	PM	1.5971*	0.7828	<0.0001	0.0325	0.1257	0.5975
	EC	1.7699*	0.8332	<0.0001	0.1357*	0.8507	<0.0001
	BS	0.3143	0.8316	<0.0001	0.0286	0.3288	0.1569

* Significantly different at $P = 0.05$ from value of BS

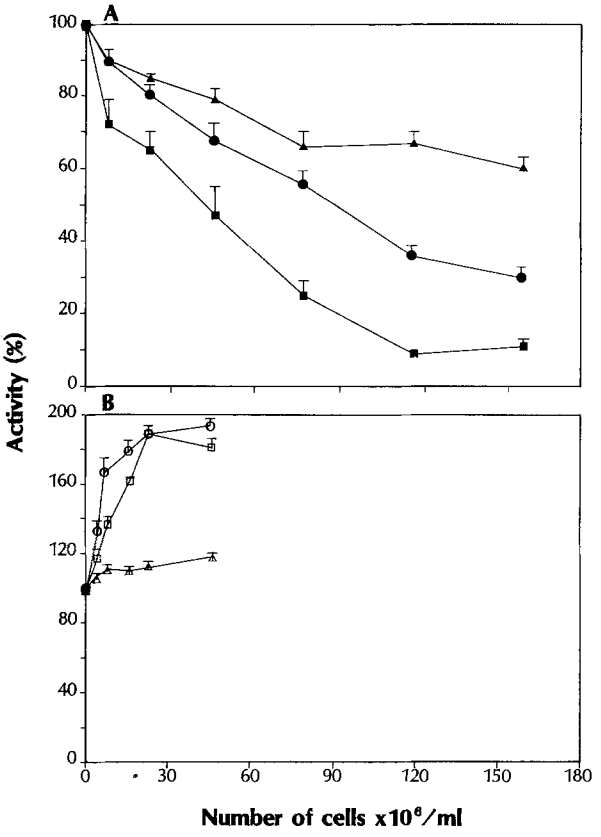

Fig. 1A, B. The effect of different quantities of bacterial cells on A UK and B SA activity: *P. mirabilis* (●, ○); *E. coli* (■, □); *B. subtilis* (▲, △)

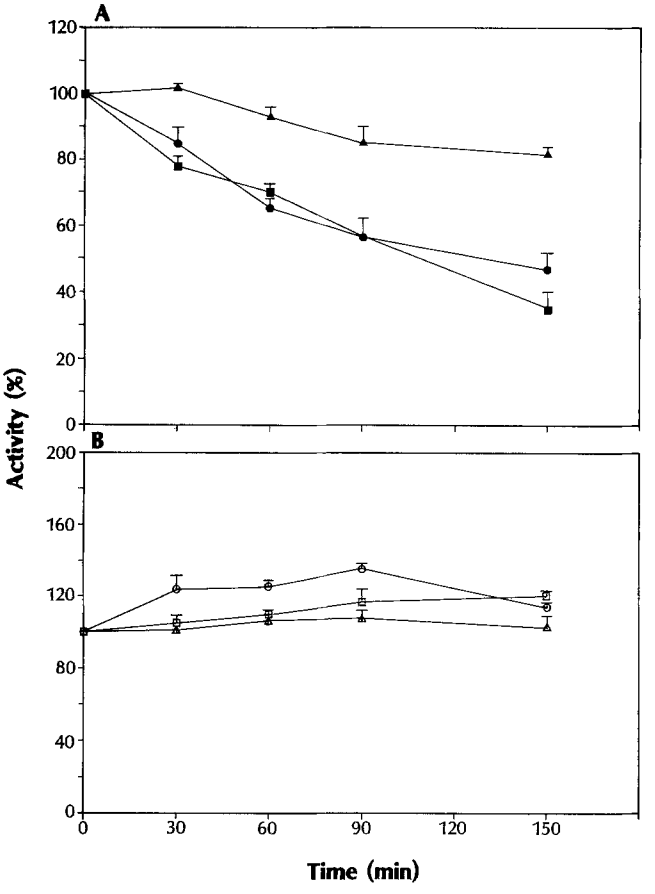

Fig. 2A, B. The effect of a constant quantity of microorganisms on A UK and B SA activity at different intervals of incubation. Inocula of 46×10^6 and 8×10^6 cells/ml were used for the UK and SA assays respectively. *P. mirabilis* (●, ○); *E. coli* (■, □); *B. subtilis* (▲, △)

Table 4. The effect of eight different bacterial species on UK and SA activity after 90 min of incubation

Bacterial species (and Gram reaction)	Activity (%)		Urease [3]	Present with stones [1, 2]
	UK	SA		
Control	100	100		
Escherichia coli (−)	47 (9)	137 (4)	−	+
Pseudomonas aeruginosa (−)	73 (2)	151 (6)	+, −	+
Proteus mirabilis (−)	68 (9)	162 (7)	+	+
Micrococcus luteus (+)	97 (1)	104 (1)	+, −	−
Bacillus subtilis (+)	80 (3)	111 (2)	−	−
Staphylococcus epidermis (+)	63 (2)	148 (4)	+, −	+
Staphylococcus aureus (+)	41 (1)	105 (1)	+	+
Enterococcus fecalis (+)	83 (1)	101 (2)	−	+

Inoculum size: UK, 46×10^6 cells/ml; SA, 8×10^6 cells/ml
Standard deviation in brackets

newly formed lactic acid and NAD^+ of the assay system after about 150 min of incubation. Consequently the NADH concentration increased and we were unable to detect spectrophotometrically the actual effect of *Proteus* cells on SA activity at this time.

According to Table 4 there was no relationship between the Gram classification of the cells or their ability to produce urease and the presence of renal stones. However, there was a strong correlation between the bacteria present with renal stones and increased levels of inhibition

or stimulation of UK or SA activity, respectively. In contrast, those bacteria not normally present with renal stones had little or no effect on these enzymes.

As renal stone formation is a complex multifactorial disease, the relative effects of urinary calcium, magnesium, urate, and bacteria on UK and SA activity would be interesting to know. The question also arises of whether there are any synergistic effects between these different factors and their possible role in renal stone formation.

This study on bacteria-affecting enzymes present in urine may explain infection-induced stone formation and could open a completely new line of research.

Acknowledgements. The authors would like to thank the Department of Physiology, University of Pretoria, for the use of their facilities. We are also grateful for services rendered by Mr. J. van Rensburg. This work was supported by Wellcome SA, the University of Pretoria, and the Medical Research Council of SA.

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