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Sialic acid concentrations in the urine of men with and without renal stones

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Summary. It has been suggested that urinary sialidase may play a role in the formation of renal stones. The present study was therefore undertaken to compare spectrophotometrically the different types of sialic acid concentrations and sialidase activities in fresh first morning urine specimens of men (21–65 years) with (13) and without (9) calcium oxalate renal stones. Although the free urinary sialic acid concentrations of the two groups of men were statistically about the same ($P=0.0614$), the total ($P=0.003$) and bound ($P=0.0012$) urinary sialic acid concentrations differed significantly. Both the total and bound sialic acid concentrations were lower in the urine specimens of the stone patients than in their healthy counterparts. This decrease in urinary sialic acid concentrations was firstly thought to be the result of elevated breakdown enzymes of sialic acid, which would favour the production of pyruvate. However, spectrophotometric determinations of the endogenous pyruvate concentrations of the two types of urine specimens did not differ significantly ($P=0.0708$). Secondly, the decrease in total urinary total sialic acid concentration of stone patients, could be attributed to less sialic acid synthesis or less renal excretion. Therefore, the same experiments were repeated using serum of 13 patients and 9 healthy men. Conversely, the total ($P=0.4425$) and bound ($P=0.2850$) serum sialic acid concentrations were found to be similar in the two types of subjects. However, the free serum sialic acid concentration of stone patients was significantly lower than in the healthy subjects ($P=0.0062$). This phenomenon is also reflected in the average ratio for serum free: bound sialic acid in healthy and stone patients, 1:7.9 and 1:18.7 respectively ($P=0.0009$). The lower free serum sialic acid concentration may lead to lower renal excretions of sialic acid. This may explain the decrease in total urinary sialic acid concentration in stone patients. The lower bound urinary sialic acid concentrations in patients was also reflected in the urinary free: bound sialic acid ratio for healthy (1:2.3) and stone patients (1:1.3). The difference between these two groups of men was highly significant ($P=0.0001$). This phenomenon might be explained by the urinary sialidase activities, which was spectrophotometrically determined at 334 nm at 37°C of 11 patients with stones and 17 healthy men. The ages of both groups of men were the same ($P=0.326$). An increase in urinary sialidase activity was observed with the stone patients ($P=0.00001$) when compared to specimens of healthy men. This might explain the decrease in urinary bound sialic acid concentration of the stone group. It seems from these results that the urinary concentration of sialic acid and the activity of urinary sialidase, may play a role in the pathogenesis of the multifactorial disease, urolithiasis.

Key words: Calcium oxalate stones – Renal stones – Serum – Sialic acid – Sialidase – Urine

In 1856 Meckel van Hemsbach stated that the first step in the pathogenesis of urolithiasis is the precipitation of a mucoid organic matrix followed by mineral deposition [10]. Since this statement, much work has been done on urinary macromolecules like Tamm-Horsfall (THM) and uromucoid. The Tamm-Horsfall mucoprotein (THM) is synthesized by the ascending loop of Henle in the nephron [8, 12], and is the major component of uromucoid [9]. Although THM has the remarkable property of gel formation [15], much controversy surrounds the possibility that urinary macromolecules consequently promote stone formation. Initially it was reported that urinary colloids had no effect on calcium oxalate crystallization [3, 20]. More recent work showed that uromucoids [4], calcium binding proteins [13] and THM [16], promote calcium oxalate and/or calcium phosphate crystal formation. Another controversy exists around the amount of mucoprotein excreted in the urine of adults with and without stones. Some reports showed that there is no difference in this excretion of uromucoids [1, 17], while other reports found that the excretion of uromucoid-rich material in calcium stone patients was greater in the stone group than in their healthy counterparts [2, 7, 9]. This controversy may be attributed to different methods of

urine storage, since it has been reported that the physical properties of the mucoprotein are altered by freezing [2]. Nevertheless, urine of black persons, who seldom develop stones, contains no uromucoid [6, 9].

The composition of urinary and matrix uromucoids is closely related and differs mainly in their relative quantities of sialic acid [6, 9]. In contrast to urinary uromucoid, which contains about 9% of the dry weight sialic acid [2], stone uromucoid contains no sialic acid. Therefore it has been hypothesized that the first step in the conversion of mucosubstances to mineralizable matrix may be the removal of the sialyl groups from uromucoid moieties. This reaction may occur in urine, because sialidase (neuraminidase) is one of the renal enzyme systems regularly present in urine [9]. If these above-mentioned theories have substance, the sialic acid concentrations should differ significantly in the urine of persons with and without stones, as well as the activity of urinary sialidase. The present study was thus undertaken to compare the sialic acid concentrations in the urine and serum of persons with and without renal stones, as well as their sialidase activity in urine.

Materials and methods

Reagents and chemicals

All reagents used were of the "Analar" grade. Boehringer Mannheim supplied NADH, rabbit muscle lactate dehydrogenase in ammonium sulphate solution, N-acetyl-neuraminic acid aldolase from *E. coli* neuraminidase (sialidase) from *Clostridium perfringens* and bovine colostrum N-acetylneuraminosyl-D-lactose (sialyllactose).

Urine specimens

First morning urine specimens were collected in plastic tubes from 13 adult male patients with calcium oxalate stones and from 9 healthy male subjects without stones. Neither group was on medication prior to the date of urine collection and none of the patients had urinary tract infections. Urine specimens obtained from the renal stone patients did not contain blood, except the last five patients listed in Table 1. However, their urine contained less than 10 erythrocytes per microlitre.

Sialic acid determinations

The working suggestion for the determination of endogenous pyruvate, free, bound and total sialic acid concentrations in urine and serum was supplied by Boehringer Mannheim. The assay consists in principle of three enzymatic reactions, namely:

a. $\text{Sialoglycoprotein} \xrightarrow{\text{Sialidase}} \text{N-acetylneuraminic acid (NANA)} + \text{acyloglycoprotein}$

b. $\text{NANA} \xrightarrow[\leftarrow]{\text{NANA-aldolase}} \text{pyruvate} + \text{N-acetylmannosamine}$

c. $\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\leftarrow]{\text{LDH}} \text{L-lactate} + \text{NAD}^+$

The measurable variable NAD^+, was determined at 334 nm. A decrease in absorbance was measured. Although reactions (b) and (c) are reversible, reaction (c) is in favour of lactate formation under the conditions to be described, and therefore, the reactions proceed virtually quantitatively in the desired direction. The reaction mixture consisted of 2.0 ml Tris-buffer (50 mM, pH 7.5), 30 µl NADH (10 mM), 50 µl urine or serum, 10 µl of freshly prepared LDH (0.2 ml LDH in 0.8 ml double distilled water), 50 µl NANA-aldolase (1 U) and 30 µl neuraminidase (180 mU). The three enzymes should not be added simultaneously to the reaction mixture. Because urine or serum contains free N-acetylneuraminic acid (NANA) and pyruvate, it is necessary firstly to determine their concentrations. Therefore, this assay starts with the addition of LDH and ends with the addition of sialidase. The initial absorbance (Ao) was measured with Tris-buffer, NADH and urine or serum in the reaction mixture. Next, LDH was added and at the end of reaction (c) the absorbance (A_1) was measured. Afterwards, NANA-aldolase was pipetted into the mixture and the absorbance (A_2) was once again measured at the end of reactions (b) and (c). In the same way A_3 was obtained at the end of reactions (a), (b) and (c) with neuraminidase. Calculations for endogenous pyruvate (A_0-A_1), free sialic acid (A_1-A_2), bound sialic acid (A_2-A_3) and total sialic acid (A_1-A_3) were done taking in consideration the dilution factor of the added enzyme. Enzyme activities were estimated at 37°C in a Hitachi 150-20 spectrophotometer connected to a data processor.

Sialic acid determinations were done on fresh urine sample of 9 men without stones and 13 male patients with calcium oxalate stones, as well as on fresh serum samples of 9 men without stones and 13 with calcium oxalate stones. The same men were used for both assays when available, but when necessary, they were supplemented with other healthy adult males or patients.

Sialidase activity in the presence of urine

The same method as described in the section Sialic Acid Determination was used for the determination of sialidase activity in the presence of 0.1 ml fresh urine with the exception of the following modifications. Sialyllactose(0.15 mM) was used as the substrate for sialidase (1.88 mU, a_m of NADH = 6,220 $M^{-1} cm^{-1}$). Both the quantities of NANA-aldolase and Tris-buffer were reduced to 20 µl (instead of 50 µl) and 1,645 µl (instead of 2,050 µl) respectively. The total reaction volume remained 2,170 µl. The sialidase activity was determined in the presence of urine obtained from 17 healthy subjects and 11 patients with stones. The same subjects were used as in previous experiments when available, but when necessary they were supplemented with other persons.

Statistical analysis

The Mann-Whitney test was used to compare the endogenous pyruvate, free, bound and total sialic acid concentrations in the urine and serum specimens of persons with and without stones. The sialidase activity expressed as a percentage of the control, in the presence of urine specimens obtained form persons with and without stones, was compared according to the Analysis of Covariance with age as covariate.

Results

The free, bound and total sialic acid concentrations in first morning urine specimens obtained from persons with and without renal stones are illustrated in Table 1 with their respective calculated average values. Although the free sialic acid concentrations in the urine specimens with and without renal stones did not differ statistically at a 5% level, a very small p-value of 0.0614 was obtained. The

Table 1. Sialic acid concentrations (mg/l) in urine specimens of men with and without renal stones (21–65 years)

Without stones						With stones					
No.	Endogenous Pyruvate	Free	Bound	Total	Free: bound	No.	Endogenous Pyruvate	Free	Bound	Total	Free: bound
1	29.29	85.96	175.93	262.81	1:2.0	1	11.51	31.16	56.47	87.97	1:1.8
2	25.11	56.95	118.37	176.01	1:2.1	2	23.01	102.82	150.95	225.21	1:1.5
3	17.78	23.64	64.07	87.97	1:2.7	3	17.78	29.99	32.58	62.99	1:1.1
4	33.47	68.77	149.87	219.37	1:2.2	4	25.10	51.41	81.45	133.58	1:1.6
5	39.75	63.40	150.95	215.03	1:2.4	5	13.60	38.56	45.61	87.71	1:1.2
6	52.30	75.22	213.94	289.96	1:2.8	6	17.78	36.41	41.27	78.19	1:1.1
7	28.24	53.73	147.70	202.00	1:2.8	7	23.01	43.91	69.50	114.03	1:1.6
8	32.43	69.84	160.73	231.3	1:2.3	8	22.00	29.99	51.04	81.45	1:1.7
9	18.83	92.41	199.82	293.22	1:2.2	9	55.44	39.63	48.87	89.05	1:1.2
Av	30.80	65.55	153.49	219.03	1:2.3	10	27.20	74.97	105.34	181.36	1:1.4
SD	10.64	20.11	44.21	62.98	0.3	11	33.47	62.12	101.00	163.99	1:1.6
						12	24.06	58.91	71.13	130.86	1:1.2
						13	18.83	47.12	43.44	91.22	1:0.9
						Av	24.06	49.77	69.13	118.9	1:1.3
						SD	10.61	20.95	33.41	53.60	0.25

Table 2. Sialic acid concentration (mg/l) in serum specimens of men with and without renal stones (21–65 years)

Without stones						With stones					
No.	Endogenous Pyruvate	Free	Bound	Total	Free: bound	No.	Endogenous Pyruvate	Free	Bound	Total	Free: bound
1	98.96	57.83	436.57	495.22	1:7.6	1	31.38	70.69	623.36	695.04	1:8.8
2	02.09	68.54	453.95	523.45	1:6.6	2	18.83	51.41	649.43	701.56	1:12.6
3	08.37	51.41	536.48	588.61	1:10.4	3	56.48	55.69	601.64	658.12	1:10.8
4	<2	40.70	417.02	458.29	1:10.2	4	115.06	83.54	605.99	690.70	1:7.3
5	81.59	64.26	538.66	603.82	1:8.4	5	29.28	36.41	584.27	621.19	1:16.1
6	43.93	72.83	543.00	616.85	1:7.5	6	29.28	34.27	642.91	677.66	1:18.8
7	50.21	81.40	551.69	634.22	1:6.8	7	71.13	38.56	399.65	438.74	1:10.4
8	69.04	66.40	584.27	651.60	1:8.8	8	25.10	10.71	532.14	543.00	1:49.0
9	46.02	85.68	445.26	532.14	1:5.2	9	108.78	27.84	534.31	562.55	1:19.2
Av	44.69	65.45	500.77	567.13	1:7.9	10	60.67	25.70	427.88	453.95	1:16.5
SD	35.15	14.13	61.77	67.14	1.7	11	0	25.00	514.76	539.76	1:20.6
						12	12	25.70	532.14	558.20	1:20.7
						13	33.47	14.99	484.36	499.56	1:32.3
						Av	45.50	38.50	548.68	587.70	1:18.7
						SD	35.40	21.40	79.00	92.60	11:2

nonsignificance may however be attributed to the low power of the test to detect differences for the given sample sizes. The power for a difference in means of 15 mg/l is only about 33%. However, the bound sialic acid concentrations between the healthy subjects and the patients ($P=0.0012$), as well as the total sialic acid concentration ($P=0.0033$), showed a statistically significant difference. The free: bound ratio was calculated and an average ratio of 1:2.3 and 1:1.3 was obtained for healthy subjects and stone patients respectively ($P=0.0001$). The mean endogenous pyruvate concentrations of 30.80 and 24.06 mg/l in the first morning urine specimens of healthy and stone subjects respectively, did not show a statistically significant difference on a 5% level ($P=0.0708$). In this case the power related to a difference of means of 10 mg/l is about 63%.

To investigate the reason why urine specimens of stone patients contained less total amounts of sialic acid than the urine of healthy persons, sialic acid determinations were done on serum obtained from male subjects with (13) and without (9) renal stones (Table 2). The free sialic acid concentration in the serum of stone patients was significantly lower than the value obtained for their healthy counterparts ($P=0.0062$). Although the bound sialic acid in serum did not differ at a 5% cut off level between the two types ($P=0.2850$), the bound sialic acid concentration tended to be higher in stone patients compared to healthy subjects. The average ratio for serum free: bound

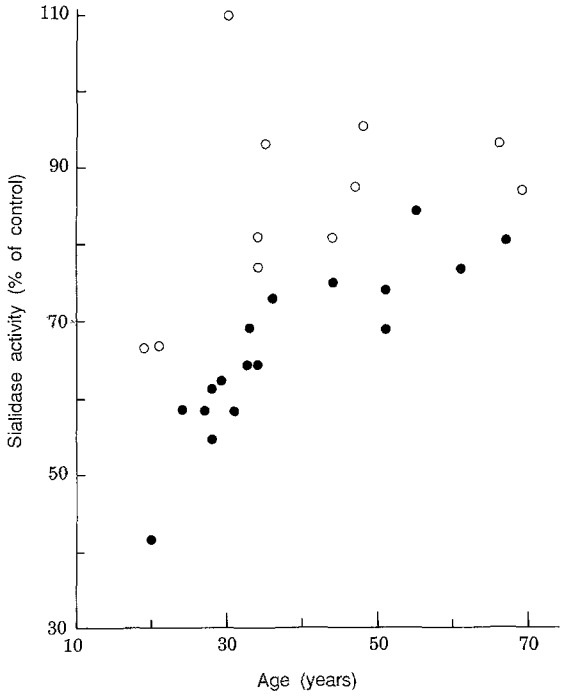

Fig. 1. Sialidase activity in the presence of fresh urine specimens obtained from 17 men without (●) and 11 persons with calcium oxalate stones (○). The conditions are described in the section Sialidase Activity in the Presence of Urine under Materials and methods. The difference in the sialidase activity of the subjects with and without stones is highly significant ($P = 0.00001$). The role of age in both groups was statistically the same ($P = 0.326$)

sialic acid in healthy and stone patients was 1:7.9 and 1:18.7 respectively ($P = 0.0009$). There was no statistically significant difference between the total serum sialic acid concentrations of the two groups of subjects ($P = 0.4425$). The differences between the endogenous pyruvate concentrations of serum obtained from subjects with and without stones were not statistically significant ($P = 0.9734$). Therefore, it does not seem possible that the decrease in free serum sialic acid in stone patients can be the result of increased serum breakdown of sialic acid.

Irrespective of the difference in sialic acid concentration between subjects with and without stones, the activity of urinary sialidase could possibly also contribute to the variations in sialic acid concentrations as observed in Table 1. Therefore, the presence or urinary inhibitors was studied next to investigate this possibility. For this purpose the known enzyme assay was modified, because the urine of both groups contained bound and free sialic acid which might have had an effect on the enzyme activities. To eliminate the effect of different urinary bound sialic acid concentrations, the assay was carried out using excess sialyllactase (0.15 mM) as described under Sialidase Activity in the Presence of Urine. The free sialic acid and endogenous pyruvate would probably not affect the assay, because the values did not differ significantly for the two groups. The results obtained with the urine of both groups are illustrated in Fig. 1. In both groups the sialidase activity was affected in the presence of urine. Higher sialidase activities were present in the stone group than in the healthy subject group ($P = 0.00001$). Although age affected the sialidase activity, the influence of age in both groups was the same ($P = 0.326$).

Discussion

Analysis of the results showed that the total urinary sialic acid concentration was significantly lower in calcium oxalate stone patients than in their healthy counterparts. In an attempt to explain this decrease in total urinary sialic acid in men with stones, the urinary endogenous pyruvate was determined firstly. The reason for this was due to the fact that it was thought that the low total urinary sialic acid concentration of stone patients was the result of increased lyase (aldolase) activity [5, 11, 19], which would favour pyruvate formation. Consequently more bound urinary sialic acid would be broken down to supply the need of free sialic acid. However, the endogenous pyruvate did not differ significantly between the two groups of men. In fact, it was even lower in the urine of stone patients than in the urine of healthy subjects. A second possible explanation for the decrease in total urinary sialic acid of stone formers may be attributed to the rate of excretion of sialic acid by the kidneys. It is generally known that free serum sialic acid is rapidly excreted in urine and that variations in the excretion rate of sialic acid could lead to serious diseases [18]. Therefore, we hypothesized that stone patients possibly excrete less sialic acid in urine than their healthy counterparts. This may in turn either be due to less sialic acid synthesis or less renal excretion. To test the sialic acid synthesis, sialic acid determinations were done on the sera of men with and without stones. The results showed a slight increase in bound serum sialic acid concentration with renal stone patients. The free serum sialic acid concentration in these patients differed significantly and was about half the value obtained in normal subjects. This decrease in free serum sialic acid with stone patients is also reflected in the serum free: bound sialic acid ratios in healthy men (1:7.9) and stone patients (1:18.7). This decrease in free serum sialic acid in stone patients could possibly lead to less renal excretion and accordingly, decreased total urinary sialic acid concentrations were observed in men with stones. This decrease in total urinary sialic acid concentration of renal stone patients may possibly cause stone formation. According to the matrix theory [10], it can be explained as follows: Regardless of the fact that renal stone patients excrete more uromucoid-rich material or THM than their healthy counterparts [2, 7, 9, 14], a decrease in the sialidation of these proteins is expected in the presence of less urinary sialic acid. Consequently, this would lead to a possible increase in mineralizable uromucoids, which would possibly result in more urinary stones.

It is interesting that the urine of adult males with stones contained significant lower bound sialic acid than the urine of healthy men. This phenomenon is also reflected in the free: bound sialic acid ratios of these two groups of men which differed significantly. The bound urinary sialic acid in healthy men was approximately double the amount of free sialic acid. Conversely, the urine of these

calcium oxalate stone formers contained about the same concentrations of free and bound sialic acid. This decrease in bound urinary sialic acid concentrations may by attributed to different sialidase activities as illustrated in Fig. 1. The sialidase activities in the urine specimens of men of the same age with and without calcium oxalate stones, differed significantly. The urine of stone patients contained higher sialidase activities than that of their healthy counterparts and therefore, lower concentrations of bound sialic acid as opposed to free sialic acid were obtained in this group. This observation supports the hypothesis that the first step in the conversion of mucosubstances to mineralizable matrix may be the removal of sialyl groups from uromucoid moieties [10], which would result in a decrease in bound sialic acid.

In conclusion, it seems as if the kidneys of men with calcium oxalate stones excrete less sialic acid than thealthy men and that the urinary sialidase activity of stone patients of different ages, is higher than the sialidase activity of healthy men of comparable ages. These results may lead to a better understanding of calcium oxalate stone formation, but the cause of this phenomenon remains unclear and urolithiasis still remains a complex multifactorial disease.

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