Determination of Urinary Oxalate with Cl^- and NO_3^- Insensitive Oxalate Oxidase Purified from Sorghum Leaf

To the Editor:

Methods for determination of urinary oxalate, which use oxalate oxidase from mosses, barley, banana peel, and beet stems, may suffer interference from physiological concentrations of Cl^- and NO_3^-, usually found in urine *(1–6)*. The usual daily excretion of Cl^- and NO_3^- are 10–15 g/day and 0.5 g/day, respectively. The potential interference of these anions is removed either by passing the urine through an ion-exchange column or by precipitation of oxalate from urine and its redissolution prior to oxalate assay *(1, 7)*. This pretreatment complicates the procedure; consequently, the sensitivity and reproducibility of the determination often suffer. In our laboratory, we have purified an oxalate oxidase from leaves of 10-day-old seedling plants of grain sorghum (CSH-5), which is insensitive to physiological concentrations of Cl^- and NO_3^- *(8)*. In the present report, we describe a new method of oxalate determination using grain sorghum leaf enzyme, which does not suffer from Cl^- and NO_3^- interference.

The seeds of grain sorghum (*Sorghum vulgare* var CSH-5) were a gift from M/s Nath Seeds Ltd. (Aurangabad, India). The 10-day-old seedling plants were raised from these seeds in the laboratory according to our method published previously *(8)*. The leaves were collected and homogenized with chilled distilled H_2O in a 1:3 ratio and centrifuged at 15 000*g* for 30 min at 4 °C. Oxalate oxidase activity present in 15 000*g* supernatant was purified to apparent homogeneity by using the combination of 0–80% $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE Sephacel, and gel filtration on Sephadex G-200 as described *(8)*. The purified enzyme gave 0.386 U/mg. One enzyme unit is defined as the amount of enzyme required to produce 1 μmol of H_2O_2/min/mL at pH 5.0 and 40 °C. The purified enzyme was stable for over a period of 1 y, when stored at −20 °C in 0.05 mol/L sodium succinate buffer, pH 5.0. Unlike moss, barley, banana peel, beet stem, and Bougainvillea leaf enzyme, sorghum leaf enzyme was unaffected by Cl^- and NO_3^- in their physiological concentration range (Table 1).

To determine urinary oxalate content, 24-h urine samples were collected from apparently healthy individuals in 2-L plastic bottles. Acidified urine (1.0 mL) was diluted with potassium phosphate buffer (0.1 mol, pH 7.0) in a 1:1 ratio, and its final pH was adjusted between 5.0 and 7.0 by HCl or NaOH. To avoid possible ascorbate interference, 200 mg of activated charcoal was added to 2.0 mL of neutralized urine sealed with Parafilm and vortex-mixed for 5 min in a 15-mL test tube. The sample was filtered through Whatman No. 1 filter paper, and filtrate was used for oxalate assay. The assay mixture, in a 15-mL test tube wrapped with black carbon paper, contained 1.7 mL of 0.05 mol/L sodium succinate buffer, pH 5.0, 0.1 mL of 0.01 mol/$CuSO_4$ solution, and 0.1 mL enzyme. This mixture was preincubated at 40 °C for 2 min. The reaction was started by adding 0.1 mL of urine (filtrate). After incubation at 40 °C for 2 min, 1.0 mL of color reagent was added. The tubes were shaken and kept at room temperature (30 ± 5 °C) for 30 min to develop the color. The A_{520} of the color was read in a Spectronic 20 (Milton & Roy), and the oxalate in the urine sample was calculated from the standard curve between oxalate concentration vs A_{520}. The color reagent consisted of 50 mg of 4-aminophenazone, 100 mg of solid phenol, and 1.0 mg of horseradish peroxidase (*RZ* = 1.0) per 100 mL of 0.4 mol/L sodium phosphate buffer, pH 7.0 *(10)* and was stored in an amber-colored bottle at 4 °C and prepared fresh every week.

A linear relationship was obtained between oxalate concentration in the

Table 1. Comparison of effects of Cl^- (as NaCl) and NO_3^- (as $NaNO_3$) on different plant oxalate oxidases.

	Cl^- effect		NO_3^- effect	
Plant source[a]	Conc.[b] added (mmol/L)	% remaining activity	Conc. added (mmol/L)	% remaining activity
All below cited sources	None	100	None	100
Mosses *(1)*	100	Inhibition		
Barley seedling *(2, 3)*	1.0	43	0.01	67
	20.0	41	0.1	39
	50.0	22	1.0	16
	100.0	15		
Banana peel *(4)*	100.0	25		
Beet stem *(5, 6)*	10.0	Inhibition	1.0	68
Grain sorghum leaf *(6)*	1.0	100	0.01	99
	5.0	100	0.1	99
	50.0	100	1.0	97
	100.0	100		
	200.0	100		
Bougainvillea leaf *(9)*	100.0	25		

[a] Numbers in parentheses are references.
[b] Conc., concentration.

reaction mixture, ranging from 0.1 to 1.2 mmol/L, with color intensity at 520 nm up to an absorbance of 0.11. The analytical recovery of added oxalate (20 and 40 nmol/sample) was 97 ± 2%. The within- and between-day CV for urinary oxalate values (n = 5) were <3.0% and <6.0%, respectively. The oxalate values in 24-h urine from apparently healthy individuals (male, n = 100) were 89–258 μmol/day, with a mean of 185.5. To evaluate the accuracy of the method, we determined the oxalate values in urine samples (n = 20) by the Sigma kit method (x) with modification *(11)* and by the present method (y). The modified Sigma kit method included the precipitation of urinary oxalate by $CaCl_2$ and ethanol and its redissolution in 0.1 mol/L HCl for removal of ion interference *(7)*, treatment with activated charcoal for removal of ascorbate interference, and measurement of H_2O_2 generated from urinary oxalate by barley oxalate oxidase in sodium succinate buffer, pH 5.0, by a color reaction using 3-methyl-3-benzothiozolinone and 3-(dimethylamine)benzoic acid and horseradish peroxidase as chromogen *(11)*. The oxalate values obtained by the two methods showed a good correlation (r = 0.9632) with the following regression equation: $y = 0.839x - 0.0025$, for a mean value of 27 ± 44 mg/L. To test the possible interference by various salts and organic substances found in urine, the following compounds were added in the reaction mixture each at two final concentrations of 0.5 and 1.0 mmol/L: NaCl, KCl, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnCl_2$, $PbCl_2$, $CdCl_2$, CH_3COOK, CH_3COONa, $NaNO_3$, Na_2HPO_4, Na_2CO_3, urea, glycine, pyruvate, glutamate, citrate, glucose, fructose, creatinine, glycollate, glyoxylate, NADH, and ascorbate. Of these compounds, only ascorbate and NADH caused 91% and 80% inhibition of the color reaction, respectively, at 0.5 mmol/L concentration, which increased to 100% at 1.0 mmol/L concentration in both the cases. Other compounds had practically no effect. NaCl had no effect, even at higher concentrations such as 50, 100, and 200 mmol/L. However, many of these substances may be present in urine at even higher concentrations in various disease states and, therefore, may need to be tested more thoroughly.

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The Mutation G_{20210}→A in the Prothrombin Gene Is a Strong Risk Factor for Pulmonary Embolism

To the Editor:

It has been reported recently by Poort et al. *(1)* that the mutation G_{20210}→A in the 3′-untranslated region of the prothrombin gene is associated with increased plasma prothrombin concentrations and venous thrombosis. The G_{20210}→A mutation was found in 18% of 28 selected patients with a personal and family history of venous thrombosis as well as in 6.2% of 471 unselected patients with a first episode of deep venous thrombosis. On the other hand, only 2.3% of 474 healthy control subjects carried the mutation. It was concluded that the G_{20210}→A mutation in the prothrombin gene is a risk factor for venous thrombosis *(1)*, which was most recently confirmed by other groups *(2–4)*.

Against this background, we addressed the question of whether pulmonary embolism as a complicated form of venous thrombosis is also associated with the G_{20210}→A transition in the 3′-untranslated region of the prothrombin gene. Therefore, we analyzed the blood samples of 27 unselected consecutive patients (11 males and 16 females; 14–78 y of age; mean age, 53 y) with pulmonary embolism for the presence of the mutation. Pulmonary embolism was related to deep venous thrombosis of the lower limbs in 10 of 27 patients (37%). For 20 patients (74%), pulmonary embolism with or without combination of deep venous thrombosis was the first event of thromboembolism, whereas the remaining 7 patients (26%) had thromboembolic events in the past. Blood samples from 245 healthy blood donors (ages 20–67 y; mean age, 45 y) were analyzed as controls. Pulmonary embolism was diagnosed by ventilation-perfusion lung scanning (n = 19), repeated perfusion lung scanning (n = 3), or clinically in combination with echocardiography and electrocardiography if unequivocal (n = 2). Questionable cases were verified by pulmonary artery angiography (n =