Determination of Nitrite and Nitrate in Stored Urine

To the Editor:

Nitric oxide ($NO^{\bullet}$) plays an important role both in maintaining normal homeostasis and in the pathogenesis of various disorders *(1)*. $NO^{\bullet}$ has a short biological half-life and is rapidly converted into its stable metabolites, nitrite and nitrate *(1, 2)*. In plasma, nitrite is rapidly oxidized to nitrate *(2)*. Determination of nitrite and nitrate (NOx) in body fluids like plasma and urine is widely used as a marker of $NO^{\bullet}$ production *(3)*. However, bacteria in urine are known to produce nitrite, and leukocytes in urine sediments contain NO synthase activity *(4)*. Therefore, it has been suggested that NOx determinations in urine are reliable only when precautions have been taken to prevent bacterial growth in the urine specimens. Indeed, Smith et al. *(4)* have shown that incubation of urine samples that contain bacteria leads to increased nitrite concentrations in these samples. Therefore, urine is often collected on ice and/or in the presence of antibiotics or organic solvents *(5–8)*. The objective of this study was to establish the effect of different storage times and temperatures on NOx concentrations in urine and to define optimal collection and storage protocols for NOx determination in urine.

NOx was determined in urine of 7 healthy volunteers and in urine of 10 individuals after kidney transplantation, as described previously *(2, 9)*, except that the final NADPH concentration was increased to 250 μmol/L to improve recovery at higher NOx concentrations. Recovery of exogenously added nitrate from five randomly selected urine samples ranged from 91% to 110% (mean, 102%) for 100 μmol/L added nitrate and from 80% to 103% (mean, 94%) for 200 μmol/L added nitrate. The mean NOx concentration in the seven healthy volunteers was 895 μmol/L (range, 533-1354 μmol/L), in accordance with previously reported values *(2, 5, 10)*. The mean NOx concentration in the 10 individuals after kidney transplantation was substantially lower (mean, 303 μmol/L; range, 55–836 μmol/L).

Within 1 h after voiding, urine samples were placed in glass tubes, capped, and incubated for 4, 8, and 24 h at 4, 20, and 37 °C. Aliquots were taken from the tubes and snap-frozen in liquid nitrogen. The NOx concentration was expressed as a percentage of the concentration measured in an aliquot of the urine sample that was immediately snap-frozen in liquid nitrogen after voiding (0-value).

Fig. 1 demonstrates that urinary NOx concentrations are stable for at least 24 h when stored at 4 °C. At 20 °C, a sudden and dramatic decrease in NOx concentration was noted in 2 of the 17 samples between 8 and 24 h of incubation. At 37 °C, markedly decreased NOx concentrations were observed in 6 of the 17 samples within 24 h. The decrease in NOx concentration was observed in normal urine samples and in urine samples from individuals after kidney transplantation.

The explanation for the decrease in NOx concentration is not clear. One possibility is the presence of bacteria that are able to reduce nitrate and nitrite. Another possibility is the release of a factor that interferes with the NOx assay, e.g., an inhibitor of the enzyme, nitrate reductase. This enzyme is necessary to convert nitrate into nitrite, which is subsequently measured in the Griess assay. To investigate this possibility, known amounts of nitrate were added to urine samples in which the sudden decrease in NOx concentration had occurred. Recovery of nitrate in these samples was near-quantitative (84–88%), suggesting that no factor is released that interferes with the NOx assay. NOx concentrations did not increase during incubation at 37 °C, compared with the 0-value in any of the urine samples tested, even in those samples with bacteriuria $\geq 10^5$ CFU/mL (10^8 colony-forming units/L). This contrasts with the finding of Smith et al. *(4)*. The reason for this discrepancy is not clear, but may be related to the fact that Smith et al. measured nitrite specifically, whereas in our study, the sum of nitrite and nitrate (NOx) was determined. Moreover, in our study only two urine samples contained $>10^8$ CFU/L, and none of our samples had urinary sediments containing leukocytes. In conclusion, our results demonstrate that NOx concentrations can be reliably determined in urine samples stored at 4 °C for at least 24 h, without addi-

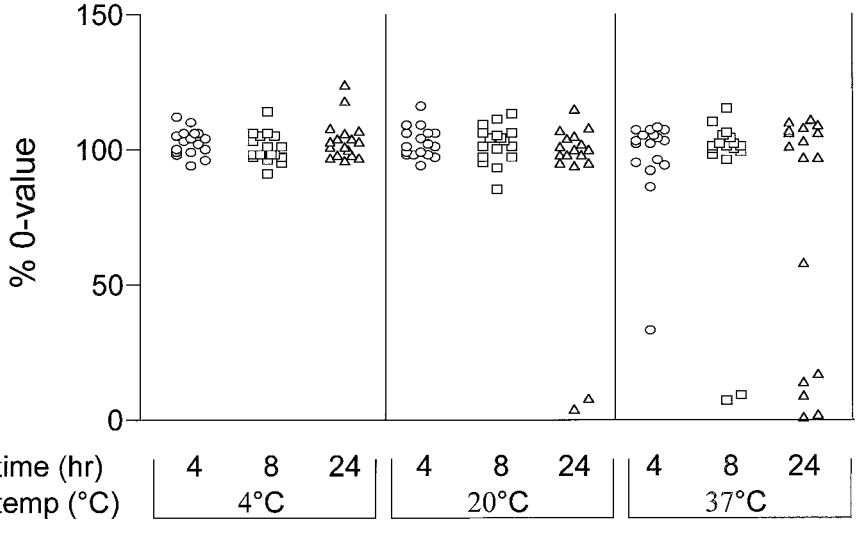

Fig. 1. Urinary NOx concentrations represented as percentage of the 0-value.

The 0-value is defined as the NOx concentration measured in the urine sample that was snap-frozen immediately after voiding. Urine samples were obtained from 7 healthy individuals and from 10 patients after kidney transplantation. Urine samples were incubated for the indicated time intervals at the indicated temperatures and subsequently assayed for NOx.

tional precautions. Serious artifacts can occur after storage >4 h at room temperature and at increased temperature, causing gross underestimation of urinary NOx concentration.

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Determination of Total Homocysteine

To the Editor:

The determination of total homocysteine (tHcy) for the diagnosis and therapy of folate and cobalamin (vitamin B_{12}) deficiencies has become an important feature of the clinical chemistry laboratory. In addition, because of the potential use of tHcy as an independent risk factor for cardiovascular disease and thromboembolism, the establishment of accurate and reliable tHcy assays has gained importance. As a result, a number of different analytical protocols to measure tHcy concentrations in human serum or plasma, using HPLC and gas chromatography/mass spectrometry (GC/MS) techniques have been described *(1)*. A recent article by Frantzen et al. *(2)* described an enzyme conversion immunoassay (EIA) for measurement of tHcy in plasma or serum. This assay is based on enzymatic conversion of tHcy (after reduction and release of endogenous homocysteine from proteins and/or disulfides) to *S*-adenosyl-L-homocysteine (SAH) by the action of SAH hydrolase (EC 3.3.1.1), followed by quantification of SAH in a competitive immunoassay with use of a monoclonal antibody against SAH. Frantzen et al. demonstrated a good method quality and showed their results obtained by the new assay to be well-correlated with a commonly used HPLC method *(3)* (as cited in *(2)*). They emphasized that the enzymatic method may serve as an alternative to HPLC analysis in clinical routines and research.

We performed a systematic comparison of the new EIA (available from Axis Biochemicals ASA, Oslo) and the GC/MS methods published by Stabler et al. *(4)* and, more recently, by our group *(5)*. We determined the concentrations of tHcy by these methods in plasma samples of 104 volunteers (ages, 20–69 years; 43 females, 61 males; tHcy range, 1.60–82.34 μmol/L) with good precision (intra- and interassay CVs were <6.2% and <8.0%, respectively, for the EIA and <3.5% and <4.8%, respectively, for the GC/MS methods). The EIA and GC/MS methods agreed well (Table 1), with results of the GC/MS methods 1% higher than the EIA results. This may be regarded as good agreement with the enzymatic method.

The new EIA has an acceptable precision and analysis range. It is quick and simple to use and can be adopted immediately by any laboratory. It appears to be an excellent choice for most routine laboratory purposes, particularly for monitoring of oral folate, betaine, and/or pyridoxine therapy, as well as for large clinical studies on the role of tHcy as a cardiovascular risk factor. On the other hand, the advantages of the GC/MS methods are, despite their cumbersomeness, their excellent lower limits of quantification (~0.2 μmol/L by GC/MS), their extended analytical range (0.2–300 μmol/L by GC/MS vs 2.0–50 μmol/L by EIA), and the possibility to determine simultaneously other metabolites of homocysteine turnover, e.g., cystathionine *(4)* and/or the other sulfur-containing amino acids, cysteine and methionine *(5)*. The latter is especially required when performing studies that aim at the response of tHcy concentrations to an oral methionine challenge in several metabolic disorders and diseases *(6, 7)*.

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