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Determination of Inorganic Nitrate in Serum and Urine by a Kinetic Cadmium-Reduction Method

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Nitrate in serum and urine was assayed by a modification of the cadmium-reduction method; the nitrite produced was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine. After samples were deproteinized with Somogyi reagent, the nitrate was reduced by Cu-coated Cd in glycine buffer at pH 9.7 (2.5 to 3 g of Cd granules for a 4-mL reaction mixture). The reduction followed pseudo-first-order reaction kinetics, a convenient time interval for assay being 75 to 90 min. Maximum reduction (85%) occurred at about 2 h. Detection limits in urine or serum were 2 to 250 μmol/L. This method does not require the reaction to go to completion, does not require expensive reagents or equipment, and can assay several samples simultaneously. Repeated assays of two serum pools gave CVs of 9.0% and 4.7% for nitrate concentrations of 31.4 and 80.2 μmol/L, respectively (n = 20 each). The mean concentration of nitrate was 1704.0 ± 1294 (SD) μmol/L (n = 21) in untimed normal urine, 81.8 ± 50.1 μmol/L in serum of 38 renal dialysis patients, and 51.2 ± 26.4 μmol/L in serum of 38 controls.

Little appears to be known about nitrate concentrations in blood in normal and pathological conditions. Nitrate itself is not toxic, but it has been implicated in the production of methemoglobinemia through reduction to nitrite (*1–3*). Nitrite also has been shown to produce carcinogenic nitroso compounds (*4, 5*), e.g., from salivary nitrate that is reduced to nitrite by oral bacteria and then swallowed. Epidemiological studies have suggested an association between nitrate ingestion and increased incidence of stomach and esophageal cancers (*6, 7*).

It has been known for many years that the body produces more nitrate than is ingested from the diet (*8–10*). This is not entirely due to the activity of intestinal flora, because germ-free rats have also been shown to excrete more nitrate than they ingested (*11–13*). Iyengar et al. (*14*) reported recently that nitrate and nitrite can be synthesized by macrophages. Thus nitrate production appears to be a mammalian process.

Nitrate in biological fluids has been determined by colorimetric procedures, either by direct nitration, or by oxidation of an organic compound to produce a color (*15, 16*). These methods in general lack specificity and are subject to interferences from biological material. Enzymatic and ion-chromatographic methods (*17–20*), although more sensitive and specific, require expensive reagents or equipment. Reduction of nitrate to nitrite by a metal, with subsequent determination of nitrite by diazotization, has been more common because it is sensitive, specific, and inexpensive (*3, 21, 22*). Drawbacks of this method have been that some metals carry the reduction further than nitrite, and that some constituents of biological material (e.g., ascorbate and phosphate) interfere.

Here we have developed a kinetic method in which nitrate is reduced to nitrite by copper-coated cadmium granules (*23*). We compared its effectiveness with that of reduction by pure Cd and by "spongy" Cd (*21*), which consists of coupled Cd and Zn. The effect of inhibitors was overcome by use of standard addition and by precipitation with zinc hydroxide. This simple and inexpensive method can be performed in any clinical laboratory, and is here applied to determine nitrate in the serum of normal and pathological individuals.

Materials and Methods

Materials

Cadmium granules. We obtained granules from Fluka Chemische Fabrik AG, Buchs, Switzerland. Using a wire cutter, we broke them into smaller pieces (20 to 40 mg). Stored in 0.1 mol/L H_2SO_4 in an Erlenmeyer flask, they are stable for at least nine months.

Glycine–NaOH buffer. Dissolve 15.0 g of glycine (Sigma Chemical Co., St. Louis, MO) in de-ionized distilled water, adjust pH to 9.7 with 2 mol/L NaOH solution, and make up to 1 L. This is stable for one month at 0 to 8 °C.

Sulfanilamide. Dissolve 5 g of sulfanilamide in 500 mL of warm 3 mol/L HCl solution, then let cool. This is stable for one year at room temperature.

N-*Naphthylethylene diamine.* Dissolve 50 mg of *N*-naphthylethylene diamine in 250 mL of distilled water. This is stable for two months at 0 to 8 °C.

Standards. Prepare working standards by diluting stock 0.1 mol/L solutions of $NaNO_2$ or KNO_3 (dissolved in 10 mmol/L $Na_2B_4O_7$) on the day of use. Stock standards are stable for at least nine months at room temperature, working standards for three days.

Specimens. Blood from renal patients was obtained before dialysis at the American University Medical Center. Blood and random (untimed) urine from apparently healthy individuals served as controls. Blood was centrifuged within 1 h of venipuncture and the separated serum was stored at −18 °C until used. Urine was stored with sodium borate (~0.5 g of $Na_2B_4O_7 \cdot 10\ H_2O$ per liter) at 0–8 °C. Specimens were stable for at least one month.

Serum pools were prepared from nonicteric, nonlipemic serum. The pool was frozen, thawed, and centrifuged at 5000 × *g* to remove insoluble material. To one half of the pool we added KNO_3, to increase the nitrate concentration by 50 μmol/L. The two halves were then subdivided into aliquots of approximately 1 mL, which were stored frozen until used.

Methods

Deproteinization (24). Add 0.5 mL of serum (or urine diluted 10-fold) to 2.0 mL of 75 mmol/L $ZnSO_4$ solution, then add, with mixing, 2.5 mL of 55 mmol/L NaOH re-

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agent. The final pH should be between 7.0 and 7.5. Let stand for 10 min and centrifuge.

Activation of cadmium granules. Rinse the acid from the granules three times with de-ionized distilled water. Swirl the granules for 1 to 2 min in a 5 mmol/L $CuSO_4$ solution in glycine–NaOH buffer. Drain, then rinse the $CuSO_4$ three times with glycine buffer. Drain and use the copper-coated granules within 10 min. Prolonged exposure of the granules to air diminishes their reductive ability. After use, rinse the granules and store them in 0.1 mol/L H_2SO_4 solution. They can be regenerated by repeating the Cu-depositing step described.

Nitrate assay. Label five 25-mL Erlenmeyer flasks, blank, sample, and standards 1 through 3. Add 1 mL of glycine–NaOH buffer to each. Add 1 mL of deproteinized sample to each of the sample and standard flasks. Add 0.5, 1.0, and 1.5 mL of 10 μmol/L KNO_3 reagent to the flasks for standard 1, standard 2, and standard 3, respectively. Adjust the volume in all the flasks to 4.0 mL with de-ionized distilled water. Start the reaction by adding with a spatula 2.5 to 3 g of freshly activated cadmium granules and stir once by swirling. Exactly 90 min later transfer 2.0 mL from each flask to an appropriately labeled tube for nitrite determination. Nitrite is determined by adding H_2O to blank, standard, or deproteinized sample to yield a volume of 2.5 mL. Add 1 mL of sulfanilamide solution, followed by 1 mL of *N*-naphthylethylenediamine (*16*). Mix, then read absorbances (*A*) against the blank at 545 nm after 20 to 60 min. A plot of A_{545} against the concentration of nitrate added should give a straight line. (Linear-regression analysis should give a coefficient $r > 0.98$.) Extrapolation to zero absorbance yields negative value of the nitrate plus nitrite concentration in the deproteinized sample (Figure 1). Calculate the nitrate concentration in the sample by subtracting the measured concentration of nitrite and multiplying by the dilution factor.

Creatinine in serum was determined by a kinetic alkaline picrate method (*25*) with an Hitachi 705 chemistry analyzer (Boehringer Mannheim, Mannheim, F.R.G.).

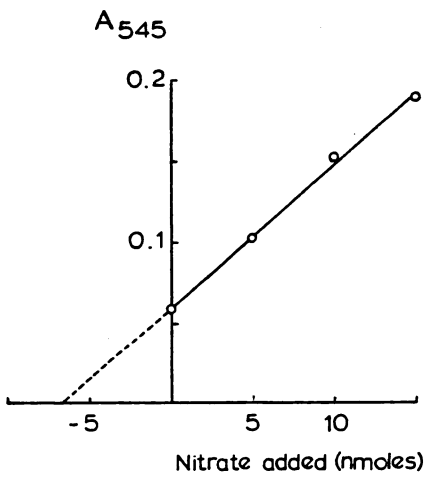

Fig. 1. Use of internal standards in the determination of serum nitrate

Each point was obtained from a reaction mixture containing 2.5 to 3 g of Cu-coated Cd granules, 200 μmol of glycine–NaOH (pH 9.7), and 1 mL of a 10-fold-diluted deproteinized sample plus the indicated amount of nitrate standard in a total volume of 4 mL. In this example, linear-regression analysis resulted in the equation $A_{545} = 0.0598 + 0.00886x$ ($r = 0.99$). Extrapolation to zero absorbance gave a value of 67.5 μmol/L for the sample

Results

We compared the ability of different forms of Cd to reduce nitrate to nitrite, both aerobically and in an atmosphere of N_2. Figure 2 shows that Cu-coated Cd was more effective than either "spongy" or pure Cd in producing nitrite. Nitrate reduction was more rapid anaerobically with all types of Cd. However, continuous bubbling of N_2 during the reaction inhibited nitrite production completely. This indicated that a gas was involved either as an intermediate, or as the ultimate product. Thus nitrite was more stable in air, and analyses were performed with air as the gas phase.

At least 2.5 to 3 g of Cd granules per reaction flask was required for maximum nitrite production. Larger amounts of Cd had no significant effect. The reaction showed a broad working pH range with a peak at about pH 9.7. This value agrees with that obtained by Follet and Ratcliff (*21*) with "spongy" Cd. The greatest percent reduction was obtained with NH_4Cl buffer; however, this buffer gave a high reagent blank: nitrite production in the absence of added nitrate was equivalent to 5 μmol/L in 90 min. We also observed spurious nitrite production with NH_4Cl buffer with "spongy" Cd. Borate buffer alone, on the other hand, prevented nitrite production but had no inhibitory effect in the presence of glycine.

The reduction of standard solutions of KNO_3 by Cu-coated Cd is shown in Figure 3. Nitrite production was proportional to nitrate concentration up to 250 μmol/L and until 120 min after the start of the reaction. The sensitivity was 1 to 2 μmol/L.

As Figure 4 shows, the rate of the reaction decreased exponentially with time, so that pseudo-first-order reaction kinetics (*26*) apply, according to the equation

$$\ln(C_i - C_t) = -kt + \ln C_i$$

where C_i is the initial nitrate concentration (equivalent to the nitrite concentration at the completion of the reaction, at infinite time), C_t is the nitrite concentration at time t, and k is the rate constant of the reaction.

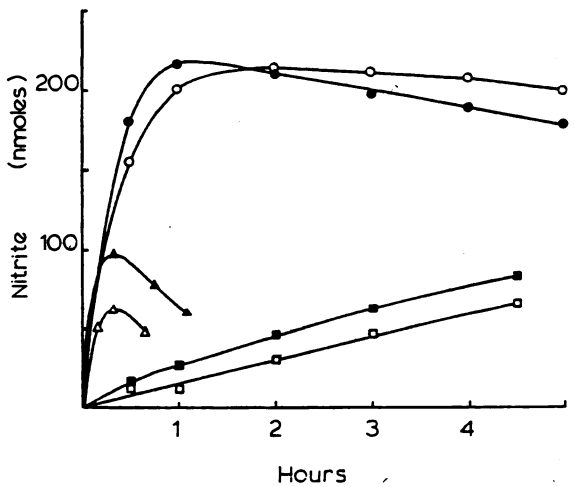

Fig. 2. Nitrite production from nitrate by different forms of Cd

The 4-mL reaction mixture contained 200 μmol of glycine–NaOH (pH 9.7), 250 nmol of KNO_3, and 2.5 to 3 g of Cd as indicated: (●, ○) Cu-coated Cd; (▲, △) "spongy" Cd; (■, □) pure Cd. *Black symbols:* under nitrogen; *white symbols:* in air. "Spongy" Cd was prepared by adding a $CdCl_2$ solution to metallic Zn granules (*21*)

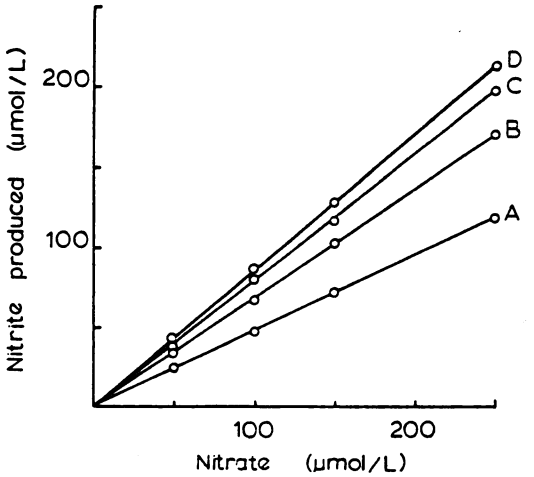

Fig. 3. Nitrate calibration curve after different time intervals

Each reaction flask contained 200 μmol of glycine–NaOH (pH 9.7), KNO_3 standard of the indicated concentration, and 2.5 to 3 g of Cu-coated Cd to start the reaction. *A*, at 30 min; *B*, 60 min; *C*, 90 min; *D*, 120 min

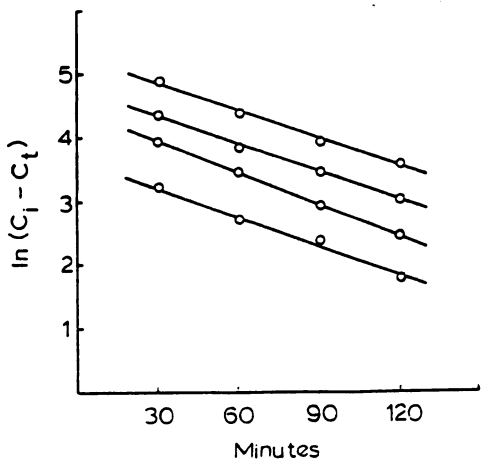

Fig. 4. Application of first-order reaction kinetics to the data in Fig. 3

Linear-regression analysis for the four concentrations shown gave a coefficient of correlation (r) >0.99 for each line

The mean slope of the regression lines, which is a measure of the rate constant, was -0.0151 ± 0.00104 min^{-1}, with a coefficient of variation (CV) of 6.9%.

The inhibitory effect of some ionic species on the reduction of nitrate by Cu-coated Cd is shown in Table 1. Ascorbate and phosphate were the more powerful inhibitors found in biological material. In contrast, the following had no observable effect on nitrate reduction: $(NH_4)_2SO_4$, 20 mmol/L; $CdCl_2$, 20 mmol/L; $Na_2B_4O_7$, 25 mmol/L in the presence of glycine; sodium citrate, 25 mmol/L; glucose, 25 mmol/L; NaCl, 40 mmol/L; urea, 250 mmol/L. Ferric salts (>0.1 mmol/L) caused an apparent increase in nitrite concentration by interfering with the diazotization reaction. Protein also interfered with diazotization by causing turbidity. In our experience deproteinization with Ag_2SO_4 (*27*) was not complete, and $K_4Fe(CN)_6$ with $ZnSO_4$ (*28*) removed part of the nitrate. The Somogyi reagent not only gave complete recovery of added nitrate and nitrite, but also removed added phosphate (10 mmol/L) ascorbate (2 mmol/L), or iron as $FeNH_4(SO_4)_2$ (1 mmol/L). The Somogyi reagent, which is known to remove phosphates and reducing substances, has been used successfully by others (*22*, *29*).

Table 1. Inhibitors of Nitrate Reduction by Cu-Coated Cd

Inhibitor[a]	Inhibition, %
$ZnSO_4$, 5 mmol/L	8.3
$CuSO_4$, 2.5 mmol/L	12.0
$CuSO_4$, 5 mmol/L	29.4
K_2HPO_4, 5 mmol/L	52.0
Ascorbate, 0.25 mmol/L	4.7
Ascorbate, 1.25 mmol/L	24.3

[a] Concentrations in the reaction mixture.

The method of standard addition was used to overcome the effect of inhibitors. When the reaction was allowed to proceed for a defined period of time, nitrite production was proportional to the total nitrate concentration, both in the presence or absence of inhibitor (Figure 5). Thus a plot of nitrite produced (y) against nitrate added (x) gave a straight line having the equation $y = A + Bx$, in which the slope, B, is the fraction of nitrate reduced. Extrapolation to $y = 0$ gives the negative value of the initial nitrate concentration in the sample. In the example shown in Figure 5, values of 20.1 and 19.5 mmol of nitrate per liter of sample were obtained in the absence and presence, respectively, of phosphate for a solution containing nitrate 20 mmol/L.

The effect of dilution was investigated with a urine specimen to test for the presence of unknown inhibitors that might influence the rate of nitrate reduction. Dilutions between five- and 100-fold had no significant effect on the nitrate concentration obtained. We compared our kinetic Cd-reduction method with the enzymatic method of Schild and Klemme (*18*) for urine specimens. Linear-regression analysis gave the equation Cd = 1.07 enzymatic − 12.0 (r = 0.986, n = 21). Interassay precision for two serum pools gave values of 31.4 (SD 4.7) and 80.2 (SD 3.6) μmol/L with CVs of 9.0% and 4.7, respectively (n = 20 in each case). Repeated assays of a urine specimen gave a mean of 885.6 μmol/L (SD 84.9, n = 15). When tested without deproteinization on three separate occasions, the same urine specimen gave a mean of 825.7 (SD 33.7) μmol/L. A survey of untimed urines from 21 apparently healthy adults gave a mean of 1704.0 (SD 1294, range 342–5392) μmol/L.

We applied the method to serum from 38 renal dialysis

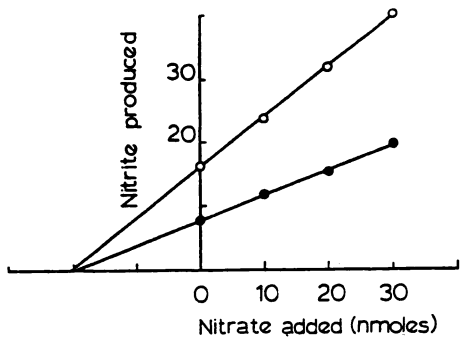

Fig. 5. Use of standard additions to obviate the effect of inhibitors

All flasks contained 20 nmol of KNO_3 plus the indicated additions: (●) phosphate, 5 mmol/L; (○) no phosphate added. The reaction time was 75 min. See *Materials and Methods* for other conditions. Although nitrite production was less in the presence of inhibitor, extrapolation to A_{545} = 0 gave essentially the same nitrate value as that without inhibitor

Table 2. Nitrate Concentrations in Serum from Healthy Adults

Nitrate, μmol/L	No. of specimens	Method	Reference
15 to 60	?	Cu-coated Cd column	*30*
37.7 (15.4)[a]	40	Cd column	*3*
36.0 (17.0)	40	Ion chromatography	*19*
34.0 (1.0)	9	HPLC	*20*
51.2 (26.4)	38	Cu-coated Cd, kinetic	This paper

[a] SD given in parentheses.

patients and obtained a mean nitrate of 81 (SD 50.1) μmol/L before dialysis. Included were 13 patients with chronic glomerulonephritis, 12 with pyelonephritis, six with diabetic nephropathy, five with malignant hypertension, and two with unknown etiology. The serum nitrate of 38 controls was 51.2 (SD 26.4) μmol/L. Student's *t*-test showed a significant difference between the two means at the 99% confidence limits. Nitrite concentration in the 76 samples was below the detection limit (0.1 μmol/L). The nitrate concentrations of the renal dialysis patients showed no correlation with their creatinine concentrations in serum.

Discussion

Cadmium has been used frequently in the determination of nitrate, apparently because of its efficiency of reduction, especially when coupled with other metals such as Zn or Cu. None of these various forms of Cd have been compared; however, our study has shown that Cu-coated Cd is the most efficient of the three. We have not been able to confirm claims that reduction is complete, and that recovery of nitrate as nitrite can be virtually 100%. The kinetic method of standardization has therefore a definite advantage in that it is not necessary for the reduction to go to completion. In their automated method for nitrate, Green et al. (*30*) used a Cu-coated Cd column preceded by a clean-up column of Dowex-50. This cation-exchange resin does not remove interference by phosphate or ascorbate, which should result in low nitrate values. We have overcome the effect of inhibitors by use of standard addition to the sample. The main function of the Somogyi reagent is to overcome protein interference in the diazotization reaction, but it provides additional protection by removing inorganic phosphate, ascorbate, and iron. The use of reaction flasks instead of a column also has the advantage that many samples can be analyzed simultaneously.

Table 2 lists the reference values for nitrate in serum in the literature, of which we are aware. Our kinetic Cd-reduction method gave serum values slightly higher than those previously reported. Methodological as well as geographical factors may account for these differences.

Although our renal dialysis patients had a significantly higher concentration of nitrate in serum than did the controls, there was no correlation between nitrate and creatinine concentrations. This suggests a predominantly tubular excretion of nitrate. Our urinary nitrate values had a wide range, among the highest reported (*8, 10, 19, 20, 27*).

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