Enzymatic Oxalate Determination in Urine

Gerda G. Mayer, Deborah Markow, and Frieda Karp

A method depending upon the use of oxalic decarboxylase is described for the determination of oxalic acid in urine. The urinary oxalate excretion of 25 healthy persons measured by this procedure is found to average 20.5 mg. (COOH)₂/24 hr. In 70 patients with calculus disease the oxalate titer of the urine was within the normal range.

The accurate measurement of oxalate concentration in urine presents considerable difficulties. The methods available in the literature are based upon either the direct formation of calcium oxalate precipitate and subsequent oxidimetric titration (1-3) or upon a preliminary ether extraction followed by a titrimetric procedure (4, 5). In a more elaborate method, the extraction with ether is combined with a colorimetric determination (6). Perhaps the most reliable approach devised to date employs radioactive-labeled oxalic acid (7), but this procedure involves special equipment not available in many laboratories.

This report describes a rapid method for the determination of oxalic acid in urine utilizing the oxalic decarboxylase from the wood-rot fungus Collybia velutipes and originally suggested by Dr. H. Shimazono. Oxalic acid is stoichiometrically converted to formic acid and CO₂ according to the reaction (8):

\[
\text{COOH} \quad | \quad \text{HCOOH} + \text{CO}_2
\]

From the Department of Urology, Columbia Presbyterian Medical Center, New York 32, N. Y.

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The chief advantage of this procedure lies in its complete specificity for oxalic acid. Recently it has been applied by Crawhall (9) and Haas (10) to assay oxalic acid in plasma and in beer. In the present paper we show that this enzyme may be used for accurate measurement of oxalic acid in urine.

Materials and Methods

Enzyme

Mycelia of *Collybia velutipes* (Strain S*) (11) is prepared as described by Shimazono and Hayashi (8) and the extract is purified through the dialyze of their "first acetone fraction." The enzyme solution may be stored at \(-15^\circ\) in 0.02 M potassium acetate buffer at pH 4.5 for a maximum of 3 weeks with no noticeable loss of activity. The enzyme preparations were assayed and an amount was used sufficient for complete decarboxylation of 10 \(\mu\)M of potassium oxalate within approximately 30 min. Six different preparations of the oxalic decarboxylase gave activities ranging from 2.7 to 4.2 U./ml., when the unit is defined as that amount which catalyzes the formation of 1.0 \(\mu\)M of CO\(_2\) per minute employing the "standard assay" procedure as described below. These activities were adequate for the analysis of urine specimens with a wide range of oxalate concentrations. The course of the reaction when 10 \(\mu\)M of potassium oxalate was placed in the Warburg flasks is shown in Fig. 1.

Treatment of Urine and Preliminary Stages

The 24-hr. collection of urine was preserved under toluene. However, regardless of how the urine is collected and stored, it has not been possible to prevent a moderate increase in oxalate detectable after urine was stored. The production of oxalate in whole urine is diminished but not completely prevented by storage of the specimens at 4\(^\circ\), and even a temperature of \(-15^\circ\) is found insufficient to prevent the change completely. Addition of up to 5 ml. of concentrated HCl per 1000 ml. is also ineffective. The amount of oxalate formed on standing in the cold is small but appreciable; therefore for accurate estimation of urinary oxalic acid excretion it is desirable that the specimens be assayed as soon as possible after collection.

The procedure used in our laboratory was as follows. The oxalic acid in a 50-ml. aliquot of a 24-hr. collection of urine was precipitated

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*Obtained as A.T.C.C. 13547 from the American Type Culture Collection, Washington 7, D.C.
at a pH of 5.0–5.2 by the addition of 2.0 ml. of 5% (w/v) CaCl₂. After this addition, the urine was usually allowed to stand a minimum of 3 hr. at room temperature and was then kept overnight at 4°. The sample was centrifuged at 2400 rpm for 30 min. at 0°. The supernatant was carefully decanted and allowed to drain. The precipitate need not be washed. Losses by washing which were shown to be appreciable are thus avoided. The precipitate was dissolved in 10 ml. of 0.1 M potassium citrate buffer pH 3.2. This procedure required at least 1 hr., with gentle heating of the suspended precipitate in a water bath at 70–80°. The volume was then readjusted to 10 ml. with water.

Assay

A 2.0-ml. aliquot of the 10-ml. solution is introduced into a Warburg flask containing 400 μM potassium citrate (pH 3.0), 6 μM ethylenedinitrilotetraacetic acid disodium salt (EDTA), and 0.1 ml. of enzyme preparation (side arm) in a total volume of 3.0 ml. Each assay consists of the sample, a standard (10 μM of potassium oxalate, pH 3.0), and a flask containing both sample and standard. The third flask is used for evaluation of a recovery of added oxalate and can be omitted once an average recovery rate has been established. Each assay is run in duplicate.

The reaction flasks are kept in ice until they are attached to the manometer and put in the bath at 37°. They are equilibrated for 10 min. open to the atmosphere. The manometers are then closed and equilibration continued for 5 more min., when the reaction is started by tipping the enzyme into the vessel. Readings are taken every 5 min. until the reaction is completed, usually within 1 hr.

Fig. 1. Rate of oxalic acid decarboxylation using 0.1 ml. enzyme solution.
The concentration of oxalic acid in the urine is calculated as follows.

\[
\frac{\text{mg.} \ (\text{COOH})_2}{21 \text{ hr.}} = \frac{\mu M \ CO_2 \ \text{generated} \times \text{M.W.} \ (\text{COOH})_2 \times \text{total vol.} / 24 \text{ hr.}}{10 \times \text{recovery} \times \text{volume of aliquot}}
\]

**Results**

Although the specificity of this enzyme has been demonstrated for serum (9) and beer (10), no studies have been made of its accuracy for analysis of urine. Therefore, tests were carried out using substances commonly found in urine in a quantity possibly sufficient to interfere with this assay. As we have reported previously (12), glucuronic acid has been a major concern.

Sodium glucuronate was investigated because of its co-precipitation with oxalic acid in urine upon the addition of calcium ions (13). When chromatographically pure sodium glucuronate was assayed alone and with the oxalic acid standard in concentrations up to 50 \(\mu M\) in 3 ml., the enzymatic oxalic acid assays were unaffected by the presence of glucuronate.

Uric acid was tested because of its poor solubility at pH 5.2. The addition of as much as 15 mg. of uric acid did not inhibit the rate of decarboxylation of the standard 10 \(\mu M\) of potassium oxalate nor did it depress the production of CO\(_2\) in the samples so treated. Cystine in amounts up to 12 mg. assayed under similar conditions did not interfere with the enzymatic determination. These compounds were tested because of their interference in the permanganate titration.

Phosphates are carried down with calcium oxalate precipitates. The addition of 30 mg. of phosphate in the form of KH\(_2\)PO\(_4\) inhibited the rate of reaction but permitted it to go to completion after 1\(\frac{1}{2}\) hr. Sixty milligrams of phosphate caused an inhibition, further slowing the rate of reaction and, after 2 hr., the assay had not yet been completed (Fig. 2). However, these quantities greatly exceed those entrained in the precipitate.

Oxalate recovery was tested in the presence of 2–20 \(\mu M\) of calcium and magnesium. Amounts of Pb\(^{++}\), Fe\(^{+++}\), and Cu\(^{++}\) of the same order of magnitude as found in the average urinary sample did not interfere with the recovery. The presence of up to 600 mg. of urea and of 250 mg. of dextrose in no way affected the decarboxylation reaction. All recovery experiments were done in a total volume of 3 ml.

The addition of 10 \(\mu M\) of potassium oxalate to the aliquot of urine prior to precipitation gave recoveries of 85 to 100\%. Addition of the
standard to the dissolved precipitate of urinary oxalate in the flask gave similar recoveries. However, these satisfactory recoveries are made possible by inclusion of EDTA in the reaction mixture. When EDTA was omitted, recoveries ranged from 70 to 80%. The addition of 3 μM increased the recovery to 85%. A 6-μM concentration raised the average recovery to at least 95%. EDTA has no effect on the action of the enzyme per se in pure oxalate solutions. It apparently acts on some inhibitor present in urine which is co-precipitated or entrained at pH 5.0–5.2. EDTA might prevent interference from anions (chiefly phosphates) (14), or it may compete with oxalic acid for a cation such as Ca++ or Mg++, thus liberating oxalate ion to be acted on by the enzyme.

Twenty-five healthy persons on unrestricted diets contributed 24-hr. specimens for urinary oxalate assay. The average excretion value was found to be 20.5 ± 7.9 (S.D.) mg. (COOH)₂/24 hr., with an average concentration of 1.82 mg. (COOH)₂/100 ml. In 70 patients suffering from calculus disease, oxalate excretion is not appreciably higher than in normal subjects. It averaged 23.5 ± 9.7 (S.D.) mg./24 hr., with a mean concentration of 1.05 mg. of (COOH)₂/100 ml. (Table 1). This supports the findings of Dempsey et al. (15).

Table 1. Urinary Oxalate Levels per 24 Hours in Normal and Calculus Disease Patients

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Discussion

The enzyme employed in this method is found to be specific, among urinary constituents tested, for oxalic acid. Those methods employing precipitation and subsequent oximetric titration are subject to errors from many causes. There is good reason to believe that other organic acids may co-precipitate. The Archer method (2) may overestimate the oxalic acid excretion in patients with infections of the urinary tract. The use of extraction of the urine with ether (6–18 hr.) (4, 5) is inconvenient for the handling of a large number of samples. Also, citrates seem to interfere in this procedure (4).

The oxalate concentration of average urine samples is insufficient to permit direct assay by the enzymatic technic. When assayed directly, small urine samples show so little CO₂ generation that the results are inaccurate. This is overcome by concentrating the oxalate by precipitation with CaCl₂ prior to the manometric assay.

The consistent increase in oxalic acid values found in urine after storage might be due to the presence of an urinary enzyme capable of oxidizing glyoxylic acid to oxalic acid. However, the failure of HCl or of freezing to prevent this change speaks against this. Hydrolysis of oxaluric acid is another possible source. Further studies are in process to discover the origin of the extra oxalate.

References

13. Light, I., Unpublished observations.