J. Biochem. 127, 279–287 (2000)

A Spectrophotometric Method for the Determination of Glycolate in Urine and Plasma with Glycolate Oxidase[1]

Eiko Maeda-Nakai and Arata Ichiyama[2]

First Department of Biochemistry, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192

Received September 9, 1999; accepted November 11, 1999

An enzymatic assay was developed for the spectrophotometric determination of glycolate in urine and plasma. Glycolate was first converted to glyoxylate with glycolate oxidase, and the glyoxylate formed was condensed with phenylhydrazine. The glyoxylate phenylhydrazone formed was then oxidized with $K_3Fe(CN)_6$ in the presence of excess phenylhydrazine, and A_{515} of the resulting 1,5-diphenylformazan was measured. Since glycolate oxidase also acts on glyoxylate and L-lactate, the incubation of samples with glycolate oxidase was carried out in 120–170 mM Tris-HCl (pH 8.3) to obtain glyoxylate as its adduct with Tris. The pyruvate formed from lactate was removed by subsequent brief incubation with alanine aminotransferase in the presence of L-glutamate, and α-ketoglutarate formed was converted back to L-glutamate by glutamate dehydrogenase and an NADPH generating system. Thus the specificity of the assay relies principally on the substrate specificity of glycolate oxidase, and high sensitivity is provided by the high absorbance of 1,5-diphenylformazan at 515–520 nm. Plasma was deproteinized with perchloric acid, and then neutralized with KOH. Plasma and urine samples were then incubated with ~5 mM phenylhydrazine, and then treated with stearate-deactivated activated charcoal to remove endogenous keto and aldehyde acids as their phenylhydrazones. The normal plasma glycolate and urinary glycolate/creatinine ratio for adults determined by this method are ~8 μM and ~0.036, respectively.

Key words: 1,5-diphenylformazan, glycolate, glycolate oxidase, glyoxylate-Tris adduct, oxalate.

The significant role of oxalate in urinary tract stone formation is well established inasmuch as 70% of all such stones contain calcium oxalate. Glycolate is one of the major precursors that contribute to hepatic oxalate synthesis. As to the origin of glycolate in the body, some is derived from the diet, because it is an intermediate of photorespiration and is present in significant concentrations in many plant tissues (*3*). A larger portion appears to be endogenously synthesized, although its metabolic origin remains unknown. Glycolate thus ingested or synthesized is either metabolized to glyoxylate by glycolate oxidase (GO, EC 1.1.3.1) in the liver or excreted in the urine. The glycolate content of animal tissues is far below that of plants, partly because of its metabolism and rapid urinary excretion (*3*).

The major metabolic pathways of glyoxylate in the liver include (i) conversion into glycine catalyzed by serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT)[3], (ii) reduction to glycolate catalyzed by D-glycerate dehydrogenase/glyoxylate reductase (DGDH/GR)[4] or lactate dehydrogenase (LDH), and (iii) oxidation to oxalate catalyzed by LDH (*4*). The transamination to glycine catalyzed by SPT/AGT appears to be the main route of glyoxylate metabolism, at least in humans, and the functional deficiency of this enzyme in the case of primary hyperoxaluria type 1 is characterized by a marked increase in the urinary excretion of glycolate as well as oxalate (*5*, *6*). These results suggest that glycolate and glyoxylate are interconvertible *in vivo*, probably *via* the oxidation of glycolate by GO in liver peroxisomes and the reduction of glyoxylate by DGDH/GR in the cytosol or LDH. Therefore, the accurate measurement of glycolate is indispensable not only for the differential diagnosis of hyperoxaluria syndromes but also for understanding the dynamics of glycolate and glyoxylate metabolism.

Glycolate has been measured by colorimetric (*3*, *7–9*), isotope dilution (*10*), and gas chromatographic methods (*11*). Recently, glycolate in the urine and plasma has been deter-

[1] This work was supported in part by a Grant-in-Aid for Scientific Research (C) (08670168) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-53-435-2322, Fax:+81-53-435-2323, E-mail: ichiyama@hama-med.ac.jp

Abbreviations: A_{515}, absorption at 515 nm; α_{mM}, absorbance of a 1 mM solution (extrapolated to 1 mM) determined with a microplate reader; ε_{mM}, millimolar extinction coefficient (light path: 1 cm); DGDH/GR, D-glycerate dehydrogenase/glyoxylate reductase; GO, glycolate oxidase; LDH, lactate dehydrogenase; SPT/AGT, serine:pyruvate/alanine:glyoxylate aminotransferase.

[3] This enzyme has been called serine:pyruvate aminotransferase (SPT, EC 2.6.1.51) or isozyme 1 of alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44), and both the activities have been shown to be of physiological importance (*1*, *2*). In this paper, the enzyme is referred to as SPT/AGT.

[4] This enzyme has been called D-glycerate dehydrogenase (DGDH, EC 1.1.1.29) or glyoxylate reductase (GR, EC 1.1.1.26), and both activities have been shown to be of physiological importance. In this paper, the enzyme is referred to as DGDH/GR.

mined after conversion to glyoxylate by GO. Petrarulo *et al.* successfully determined the glyoxylate thus formed by an HPLC method after derivatization to its phenylhydrazone (*12*, *13*). Although the GO-coupled HPLC method is potentially quite effective, a reliable spectrophotometric assay also deserves to be developed, since it should be practicable in every laboratory using commercially available GO from spinach. Kasidas and Rose (*14*) determined glycolate in urine and plasma by measuring H_2O_2 generated in the GO-catalyzed oxidation of glycolate to glyoxylate. In this case, however, the H_2O_2 derived from L-lactate and glyoxylate had to be corrected accurately, because GO catalyzes the oxidation of not only glycolate, but also L-lactate and glyoxylate. In another enzymatic method, the level of glyoxylate formed in the oxidation of glycolate was determined spectrophotometrically after derivatization to its 2,4-dinitrophenylhydrazone (*15*). This method is also impracticable, however, especially for the assay of glycolate in plasma, because the absorbance of glyoxylate 2,4-dinitrophenylhydrazone is not high enough (ε_{mM} at 450 nm in 0.5 N NaOH is about 8.9) and, in addition, the color of the hydrazone in basic solution is extremely unstable (*16*).

In this study, we attempted to measure glycolate by the GO-catalyzed oxidation to glyoxylate, followed by non-enzymatic conversion of the latter to 1,5-diphenylformazan *via* glyoxylate phenylhydrazone. It is known that an intensely colored 1,5-diphenylformazan (ε_{mM} at 520 nm is 40–41) is formed when the oxidative azo coupling of glyoxylate to phenylhydrazine takes place with simultaneous decarboxylation in the presence of excess phenylhydrazine (*17*) (Fig. 1). Since the color development is not entirely specific to glyoxylate (*18*), the 1,5-dipheylformazan method may not be well suited for the determination of glyoxylate in biological samples. If it is connected to the GO-catalyzed conversion of glycolate to glyoxylate and enough consideration is given to the substrate specificity of GO, however, this method would be applicable to the determination of glycolate in biological samples with sufficient specificity and sensitivity.

MATERIALS AND METHODS

Materials—Activated charcoal, Shirasagi A1, was obtained from Takeda Pharmaceutical (Osaka), and partially deactivated with stearic acid as described previously (*19*). GO from spinach, Na-glyoxylate-H_2O and Li-L-lactate were purchased from Sigma-Aldrich Japan (Tokyo), and alanine aminotransferase, glutamate dehydrogenase, glucose 6-P dehydrogenase, and beef liver catalase were from Roche Diagnostic (Tokyo), ADP, NADH, NADPH, and glucose 6-P were from Oriental Yeast (Tokyo); FMN, $K_3Fe(CN)_6$, metaphosphoric acid, Na-pyruvate, α-ketoglutaric acid, Tris(hydroxymethyl)aminomethane (Tris) and phenylhydrazine-HCl were from Wako Pure Chemical Industries (Osaka); Na-glycolate was from Nacalai Tesque (Kyoto); creatinine was from Merck (Darmstadt, Germany); Na-[1-^{14}C]glycolate (2.035 GBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA); and Ekicrodisc 13CR (a 0.45 μm polytetrafluoroethylene filter) was from Gelman Science Japan (Tokyo). Sources of the reagents used for the determination of oxalate have been described previously (*19*).

Determination of Glyoxylate after Conversion into 1,5-Diphenylformazan—Glyoxylate was quantitatively converted, *via* glyoxylate phenylhydrazone, into 1,5-diphenylformazan, and its absorbance at 515 nm (A_{515}) was measured, essentially as described by Dekker and Maitra (*20*) and Matsui *et al.* (*17*). A reaction mixture (140 μl) in a 96-well microplate initially contained 0.2–5 nmol of glyoxylate and 10 μl of 2% (w/v) phenylhydrazine-HCl. After 15-min incubation at room temperature, A_{515} (A-1) was measured with a Microplate Bio Kinetics Reader (EL 340, Bio-Tek Instrument, Winooski, VT, USA) against a control containing no glyoxylate as a blank. Then 52 μl of conc. HCl and 8 μl of 5% (w/v) $K_3Fe(CN)_6$ were added (final 200 μl) in that order with mixing after each addition, after which A_{515} (A-2) was measured at about 1-min intervals for 8–10 min. A_{515} increased sharply after the addition of $K_3Fe(CN)_6$, reached a plateau, and then decreased slowly. The difference between the peak values of A-2 and A-1 (ΔA_{515}) was linear against the glyoxylate concentration up to about 25 μM, and a_{mM} (ΔA_{515} when the concentration of glyoxylate is 1 mM) of the glyoxylate determination was about 19 under the conditions used. When the reaction was carried out in a cuvette in a total volume of 1.0 ml and A_{520} was measured with a spectrophotometer, ε_{mM} of the glyoxylate determination was about 40.

Determination of Glycolate—Glycolate was first oxidized

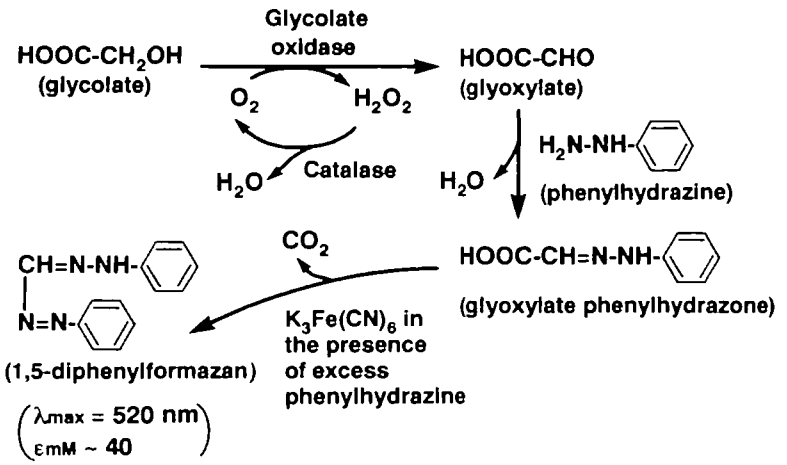

Fig. 1. **Principle of the glycolate oxidase-coupled spectrophotometric determination of glycolate.** Glycolate is first incubated with GO in 120–170 mM Tris-HCl (pH 8.3), and the glyoxylate formed is trapped as its adduct with Tris. Catalase is included in the reaction mixture to protect glyoxylate from decomposition by H_2O_2. The accumulated glyoxylate as the Tris adduct is derivatized to its phenylhydrazone, and then converted into intensely colored 1,5-diphenylformazan by oxidation with $K_3Fe(CN)_6$ in the presence of excess phenylhydrazine.

to glyoxylate in Tris-HCl (pH 8.3) with GO, and the glyoxylate-Tris adduct was quantified after conversion to 1,5-diphenylformazan. Figure 2 shows the reaction system for glycolate determination. For every sample, four control incubations, (1), (3), (4), and (5), were carried out. In (1), 8 µl of 3.2 M $(NH_4)_2SO_4$ and 16 µl of 1 mM FMN were added instead of 8 µl of ~20 units/ml GO in 3.2 M $(NH_4)_2SO_4$ containing 2 mM FMN (minus GO control). In (3), (4), and (5), 1.5, 3.0, and 4.5 nmol of glycolate were added, respectively, in addition to the sample, to measure the efficiency of each determination. Another set of incubations contained no sample and was referred to as "minus sample control." The concentration of Tris buffer (pH 8.3) was 120–170 mM to trap glyoxylate formed as an adduct with Tris, and catalase was added in excess to protect glyoxylate from decomposition by H_2O_2. Each reaction was carried out in a 1.5-ml polypropylene microtube in a total volume of 233 µl at 37°C for 30 min. Then a pyruvate scavenging reagent containing L-glutamate, ADP, glutamate dehydrogenase, glucose 6-P, and glucose 6-P dehydrogenase was added together with NADPH and alanine aminotransferase to convert any pyruvate formed from L-lactate in the sample into alanine (*cf.* Fig. 8). The reactions were finally stopped by adding 70 µl of 15% (w/v) metaphosphoric acid to 280 µl of reaction mixture. The choice of 3% metaphosphoric acid to finally stop the glycolate oxidase and pyruvate scavenging reactions was according to Dekker and Maitra (*20*). In confirmation of their data, the effect of up to 4.3% metaphosphoric acid on ε_{mM} of the glyoxylate determination was minimal.

After centrifugation (13,000 rpm/10 min at 4°C) 125 µl of the supernatant was subjected to the glyoxylate assay as described above, except that the absorbance of 1,5-diphenylformazan at 515 nm was read against a blank containing all the reagents except the sample and GO or $(NH_4)_2SO_4$/FMN. Preliminary experiments showed that glyoxylate

	(1)	(2)	(3)	(4)	(5)	
1 M Tris-HCl (pH 8.3)	40	40	40	40	40	
10 mg/ml Catalase	5	5	5	5	5	
0.3 mM Glycolate	—	—	5	10	15	
Sample + H_2O	164	180	175	170	165	
3.2 M $(NH_4)_2SO_4$	8	—	—	—	—	
1 mM FMN (~ pH 7)	16	—	—	—	—	
* ~20 U/ml GO	—	8	8	8	8	
Incubation : 37°C/30 min					233 µl	
Pyruvate scavenging reagent					37	
2 mM NADPH					5	
* 0.4 mg/ml ALT					5	
Incubation : 37°C/15 min					280 µl	
15% Metaphosphoric acid					70 µl	
					350 µl	

Fig. 2. **Standard reaction system for the glycolate oxidase-catalyzed conversion of glycolate to glyoxylate in glycolate determination.** The pyruvate scavenging reagent comprised 40.5 mM L-glutamate, 6.76 mM ADP, 0.27 mg/ml glutamate dehydrogenase, 40.5 mM glucose 6-P, and 0.26 mg/ml glucose 6-P dehydrogenase. Since the GO, alanine aminotransferase, and glucose 6-P dehydrogenase preparations used were suspensions in 3.2 M $(NH_4)_2SO_4$, the concentration of NH_4^+ in the second incubation in a total volume of 280 µl was 0.43 M. Abbreviation: ALT, alanine aminotransferase.

in the form of the Tris adduct is converted into 1,5-diphenylformazan as effectively as free glyoxylate. ΔA_{515} of (1) ($\Delta A_{(1)}$) representing noise or the increase in absorbance due to compounds other than glycolate in the sample was usually very small, but $\Delta A_{(2)}$, $\Delta A_{(3)}$, $\Delta A_{(4)}$, and $\Delta A_{(5)}$ were all corrected for $\Delta A_{(1)}$. $\Delta A_{(2)} - \Delta A_{(1)}$ is the absorbance increase due to glycolate in the sample, and $\Delta A_{(3)} - \Delta A_{(2)}$, $\Delta A_{(4)} - \Delta A_{(2)}$, and $\Delta A_{(5)} - \Delta A_{(2)}$ are those due to 1.5, 3.0, and 4.5 nmol of standard glycolate in the initial reaction mixture, respectively. From these values the linearity of each determination was verified, and from the slope of the linear line a_{mM} of the glycolate determination in each sample was calculated. Only when $\Delta A_{(3)}$, $\Delta A_{(4)}$, and $\Delta A_{(5)}$ fell on a linear line and the calculated a_{mM} was above 13 was the determined $\Delta A_{(2)}$ value taken to be valid. The amount (nmol) of glycolate in the sample was then calculated using the equation,

$$[(\Delta A_{(2)} - \Delta A_{(1)})/a_{mM}] \times 1/5 \times 350/125 \times 1000$$

The glycolate assay was performed using at least two different volumes of urine or plasma samples, and only when the determined values were proportional to the amount of samples was the assay taken to be valid.

Preparation of Urine Samples for Glycolate Determination—It is highly desirable, especially for oxalate determination, that 24-h urine is collected in a bottle containing 10–15 ml of 6 N HCl, but in this study 24-h urine was collected from in-patients in our university hospital in the absence of HCl. A portion of the collected urine was mixed with one-hundredth volume of 6 N HCl as soon as possible, and stored frozen at –80°C. After thawing, the acidified urine was centrifuged wat 13,000 rpm for 10 min. The supernatant was adjusted to pH 6.0–6.5 with 1 N NaOH, the volume of which was noted, and then mixed with one-twentieth volume of 100 mM phenylhydrazine (pH 6.0), and incubated for 15 min at room temperature. Then the incubated urine sample was subjected to charcoal treatment as described previously (*19*) to remove phenylhydrazine, phenylhydrazones of endogenous keto and aldehyde acids, and interferring substances in the urine, if any. In brief, the urine sample was mixed with 80 mg/ml of stearate-deactivated activated charcoal, and, after standing for ~10 min at room temperature with occasional shaking, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was then passed through an Ekicrodisc 13CR to remove fine particles of charcoal.

The recovery of the [^{14}C]glycolate added to the urine through the entire procedures for the preparation of urine samples was almost quantitative as described under "RESULTS AND DISCUSSION". Therefore, the concentration of glycolate in the urine was calculated using the equation,

$$\mu M \text{ in urine} = \text{nmol glycolate/ml urine sample} \times 1.05(1.01 + k)$$

where k is ml of NaOH required to neutralize 1.01 ml of acidified urine to pH 6.0–6.5.

Preparation of Plasma Samples for Glycolate Determination—Morning samples of venous blood were taken under fasting conditions in the presence of heparin as an anticoagulant and centrifuged as soon as possible at 3,000 rpm for 5 min at 4°C. In one experiment, serum was separated from the venous blood of a healthy volunteer using a Venoject II autosep (Terumo, Tokyo). The plasma and serum obtained were stored at –80°C until use, deproteinized after

thawing by adding one-tenth volume of 6.6 N perchloric acid, and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was neutralized to about pH 6 with KOH, the volume of which was noted, and the K-perchlorate precipitate formed was removed by centrifugation in the cold. Then the neutralized supernatant was subjected to treatment with phenylhydrazine and stearate-deactivated activated charcoal, as in the care of urine samples.

Since the recovery of glycolate through the preparation of plasma samples was also nearly quantitative so far as the concentration of [^{14}C]glycolate added to the plasma was concerned, as described under "RESULTS AND DISCUSSION," the concentration of glycolate in plasma was calculated using the equation

$$\mu M \text{ in plasma} = \text{nmol glycolate/ml plasma sample} \times 1.05(1.10 + k')$$

where k' is ml of KOH required to neutralize 1.10 ml of deproteinized plasma to pH 6.0–6.5.

Other Methods—Urinary oxalate was determined by an enzymatic method using oxalate oxidase as described previously (*19*). Creatinine was determined by Jaffe' reaction. ^{14}C-radioactivity was determined in a liquid scintillation counter with a toluene-based emulsion scintillator containing 33% (w/v) Triton X-100, 0.5% (w/v) 2.5-diphenyloxazole (DPO), and 0.03% (w/v) 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene (dimethyl-POPOP).

RESULTS AND DISCUSSION

Determination of Glyoxylate and Glycolate after Conversion to 1,5-Diphenylformazan—Figure 3A shows the absorption spectrum of 1,5-diphenylformazan prepared from glyoxylate *via* glyoxylate phenylhydrazone as described under "MATERIALS AND METHODS." In agreement with Matsui *et al.* (*17*), an absorption maximum was observed at 520 nm with a shoulder at around 545 nm. The time course of 1,5-diphenylformazan formation was rapid, with A_{520} reaching a maximum level 2.5 to 4 min after mixing glyoxylate phenylhydrazone with $K_3Fe(CN)_6$. Thereafter, A_{520} decreased slowly (Fig. 3B). A_{520} (A_{515} when a microplate reader was used) was subsequently measured at about 1 min intervals for 8–10 min to find the maximally increased level or 3 to 4 min after glyoxylate phenylhydrazone was mixed with $K_3Fe(CN)_6$. Figure 4 shows linearity of the glyoxylate and glycolate determination after the conversion to 1,5-diphenylformazan. The standard curve for the spectrophotometric determination of glyoxylate was linear up to 40 μM glyoxylate, and the ε_{mM} of the determination was calculated to be 40–41. When glycolate was oxidized with GO to glyoxylate in Tris-HCl buffer (pH 8.3), the glyoxylate formed trapped as an adduct with Tris, and the glyoxylate-Tris adduct converted to 1,5-diphenylformazan *via* glyoxylate phenylhydrazone, the increase in A_{520} (ΔA_{520}) was linear against the expected concentrations of glycolate-derived glyoxylate. In addition, the ε_{mM} was calculated to be 40–41, suggesting that the conversion of glycolate to glyoxylate was quantitative.

Figure 5 shows the time course of glyoxylate formation when 300 nmol glycolate was incubated in 125 mM Tris-HCl (pH 8.3) with ~0.32 unit GO. The formation of glyoxylate determined as 1,5-diphenylformazan was rapid and complete after about 10-min incubation under the conditions used. In this experiment, 25.7 μM glyoxylate had been expected to be carried into the mixture if the conversion of glycolate to glyoxylate was quantitative, and a ΔA_{520}

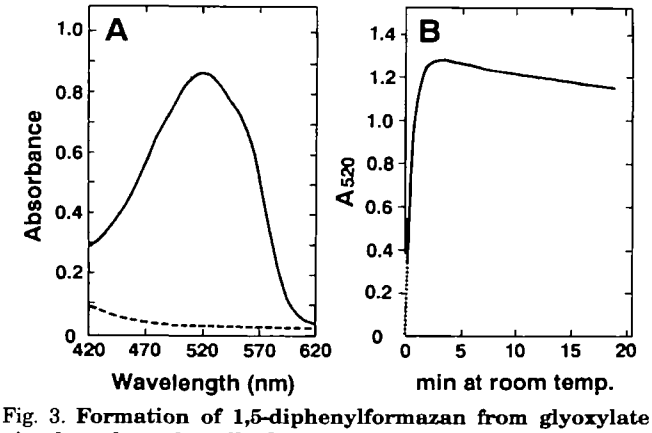

Fig. 3. **Formation of 1,5-diphenylformazan from glyoxylate *via* glyoxylate phenylhydrazone.** A: Absorption spectrum of the 1,5-diphenylformazan formed. B: Time course of 1,5-diphenylformazan formation. In this experiment, glyoxylate phenylhydrazone was prepared in a 700 μl reaction mixture in a cuvette containing 100 μl of 1% phenylhydrazine-HCl and ~20 nmol glyoxylate (A) or 100 mM K-phosphate (pH 8.3), 100 μl of 1% phenylhydrazine-HCl and ~30 nmol glyoxylate (B). After incubation at room temperature for 15 min, 260 μl of conc. HCl and 40 μl of 5% $K_3Fe(CN)_6$ were added in this order with immediate mixing after each addition. In A, the absorption spectrum was measured in a spectrophotometer against H_2O as the blank 4 min after the addition of $K_3Fe(CN)_6$, In B, the time course of changes in A_{520} was followed. Dashed line in A: absorption of a control containing all ingredients except glyoxylate (minus glyoxylate control).

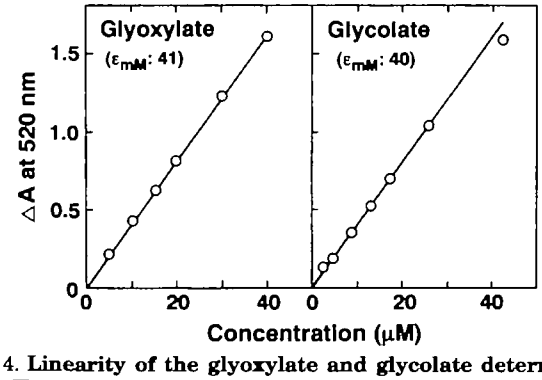

Fig. 4. **Linearity of the glyoxylate and glycolate determination.** The reaction mixture (500 μl) for the GO-catalyzed conversion of glycolate to glyoxylate comprised 70 μmol Tris-HCl (pH 8.3), 20 nmol FMN, 10 μg catalase, ~0.32 unit GO, and 2.5, 5, 10, 15, 20, 30, or 50 nmol glycolate. After 90-min incubation at 37°C, 200 μl of 15% metaphosphoric acid was added and the denatured proteins were removed by centrifugation. Then, 600 μl of the supernatant was mixed in a cuvette with 100 μl of 1% phenylhydrazine-HCl, and after 15 min at room temperature, 260 μl of conc. HCl and 40 μl of 5% $K_3Fe(CN)_6$ were added with mixing after each addition. ΔA_{520} was measured in a spectrophotometer 4 min after the addition of $K_3Fe(CN)_6$. A control incubation containing no glycolate was run simultaneously, and all experimentally determined ΔA_{520} values were corrected for the control. For the determination of glyoxylate, reaction mixtures (600 μl) containing 70 μmol K-phosphate (pH 8.3) and 5, 10, 15, 20, 30, or 40 nmol glyoxylate were mixed in a cuvette with 100 μl of 1% phenylhydrazine-HCl, and after 15 min at room temperature the glyoxylate phenylhydrazone formed was oxidized to 1,5-diphenylformazan and its A_{520} was measured as above.

of ~1.05 was obtained. The calculated ε_{mM} (40.9) was almost the same as that of glyoxylate determination (*cf.*, Fig. 4), again suggesting an almost complete conversion of glycolate into glyoxylate. In addition, almost the same ΔA_{520} was measured after incubation of glycolate with GO for up to 50 min or longer (in other experiments), indicating that the glyoxylate-Tris adduct formed is stable even in the presence of ~0.32 unit GO. Essentially the same results were obtained when 30 nmol glycolate was subjected to incubation with ~0.32 unit GO.

However, this method of glyoxylate determination is not entirely specific to glyoxylate, since pyruvate phenylhydrazone also produced a colored product with a maximum absorption at 510 nm (Fig. 6A). The absorption at 520 nm of the product from pyruvate was only about 1/30 that of 1,5-diphenylformazan, with an ε_{mM} of about 1.3 calculated 4 min after the addition of $K_3Fe(CN)_6$. In addition, this product was unstable and decreased to about 25% after 15 min (Fig. 6B). Nevertheless, the pyruvate formed from L-lactate had to be eliminated without affecting glyoxylate, because the concentrations of L-lactate and glycolate in human plasma were expected to be 0.5–1.6 mM and several μM, respectively, and GO from spinach catalyzes the oxidation of both glycolate and L-lactate.

Elimination of Pyruvate by Alanine Aminotransferase— We then examined whether or not alanine aminotransferase can be used to eliminate pyruvate without affecting glyoxylate. For this purpose, the formation of α-ketoglutarate from L-glutamate in the alanine aminotransferase reaction was coupled to the oxidation of NADH in the presence of NH_4^+ and a large excess of glutamate dehydrogenase, and the decrease in A_{340} was followed. As shown in Fig. 7, glyoxylate serves as a substrate of alanine aminotransferase in 50 mM triethanolamine buffer (pH 8.0), although the rate of the reaction with glyoxylate is much slower than that with pyruvate. When the reaction was carried out in 100 mM Tris-HCl (pH 8.3), on the other hand, glyoxylate was totally inert, whereas pyruvate was rapidly and quantitatively metabolized. In addition, when 300 nmol of glycolate was incubated with ~0.32 unit GO in a reaction mixture (500 μl) containing 140 mM Tris-HCl (pH 8.3) and the alanine aminotransferase–glutamate dehydrogenase system, glyoxylate, determined as 1,5-diphenylformazan, was formed rapidly reaching a plateau within 10 min, and then remained at almost the same level for up to 60 min. These results suggest that alanine aminotransferase hardly acts on glyoxylate-Tris adduct.

In order to eliminate pyruvate offectively, the alanine aminotransferase reaction had to be coupled to glutamate dehydrogenase whose K_{eq} ([α-ketoglutarate] [NADH]

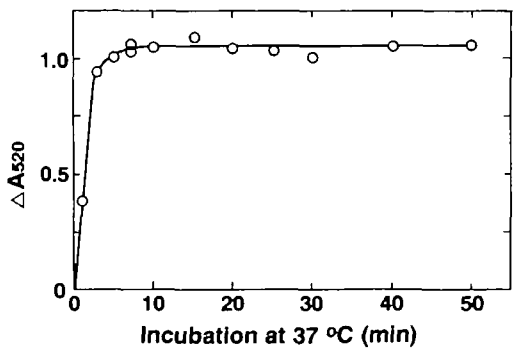

Fig. 5. **Time course of the glycolate oxidase–catalyzed glyoxylate formation from glycolate.** The reaction mixtures (560 μl) containing 300 nmol glycolate, 40 nmol FMN, 40 μg catalase, and ~0.32 unit GO in 125 mM Tris-HCl (pH 8.3) were incubated at 37°C for the indicated periods of time, and terminated by adding 140 μl of 15% metaphosphoric acid. After centrifugation at 13,000 rpm for 5 min, 60 μl supernatant was mixed with 540 μl of H_2O and 100 μl of 2% phenylhydrazine-HCl, and the mixture was incubated for 15-min at room temperature. A-1 at 520 nm was then measured with a spectrophotometer. Then 260 μl of conc. HCl and 40 μl of 5% $K_3Fe(CN)_6$ were added in this order, and A-2 at 520 nm was measured 2.5 to 5 min after the addition of $K_3Fe(CN)_6$. The increase in A_{520} by the addition of $K_3Fe(CN)_6$ (ΔA_{520}, A-2–A-1) was taken to represent A_{520} due to the 1,5-diphenylformazan formed.

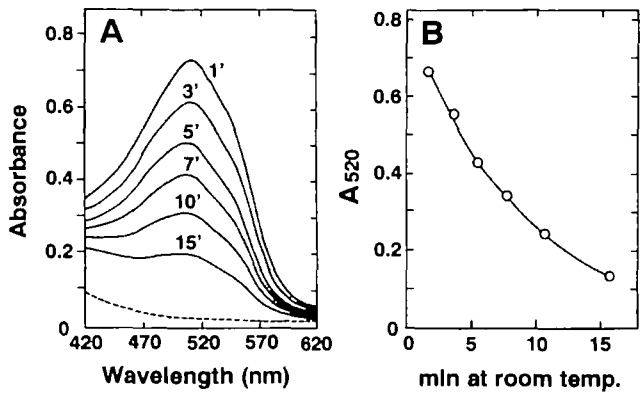

Fig. 6. **Oxidation of pyruvate phenylhydrazone with $K_3Fe(CN)_6$.** Pyruvate phenylhydrazone was prepared in a reaction mixture (700 μl) in a cuvette containing 500 nmol pyruvate and 50 μl of 2% phenylhydrazine-HCl following 15-min incubation at room temperature. Then 260 μl of conc. HCl and 40 μl of 5% $K_3Fe(CN)_6$ were added in this order, and the absorption spectrum was taken with a spectrophotometer against H_2O as the blank 1, 3, 5, 7, 10, and 15 min after the addition of $K_3Fe(CN)_6$. A: Absorption spectrum of the colored product formed by oxidation of pyruvate phenylhydrazone. Dashed line: absorption of a control containing all ingredients except pyruvate (minus pyruvate control). In B, changes in A_{520} are plotted as a function of time after the addition of $K_3Fe(CN)_6$.

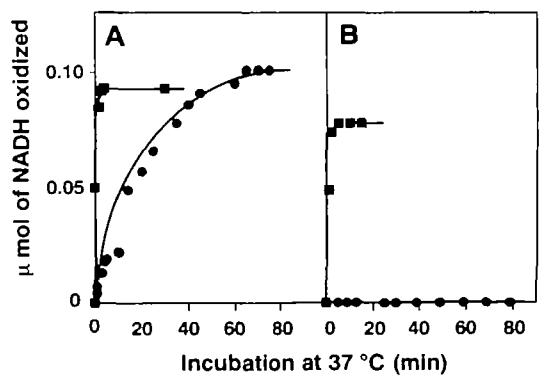

Fig. 7. **Glyoxylate as a substrate of alanine aminotransferase.** The reaction mixture (1.0 ml) in a cuvette contained 5 μmol L-glutamate, 10 μg alanine aminotransferase, 66 μmol NH_4Cl, 1 μmol ADP, 0.2 μmol NADH, 50 μg glutamate dehydrogenase, 0.1 μmol pyruvate (■) or glyoxylate (●) in 50 mM triethanolamine-HCl (pH 8.0) (A) or 100 mM Tris-HCl (pH 8.3) (B). The reactions were started by the addition of alanine aminotransferase, and the decrease in A_{340} was followed with a spectrophotometer at 37°C. The amount (μmol) of NADH oxidized was calculated on the basis of ε_{mM} of NADH at 340 nm of 6.22.

$[NH_4^+]$/[L-glutamate] $[NAD^+]$) has been reported to be 4.5×10^{-14}, because alanine aminotransferase catalyzes a reversible reaction with a K_{eq} ([L-glutamate] [pyruvate]/[α-ketoglutarate] [L-alanine]) of 2.2. Unfortunately, however, the NADH used as a substrate for glutamate dehydrogenase adversely affected the determination of glyoxylate as 1,5-diphenylformazan. Therefore, we used a low concentration (36 μM) of NADPH instead of a substrate level of NADH, and the glutamate dehydrogenase reaction was coupled to glucose 6-P dehydrogenase to generate NADPH (Fig. 8). In the presence of the glutamate dehydrogenase and glucose 6-P dehydrogenase systems with 36 μM NADPH in 280 μl of reaction mixture (*cf.* Fig. 2), the ΔA_{520} or ΔA_{515} values due to known amounts of standard glycolate were only slightly less than or almost the same as in their absence.

Preparation of Urine and Plasma Samples for the Determination of Glycolate—In our preliminary survey, EDTA levels above 10 mM caused a dose-dependent increase in A_{515} when the EDTA was mixed with phenylhydrazine and incubated with $K_3Fe(CN)_6$ in 3 N HCl, whereas heparin up to 50 units/200 μl reaction mixture had no effect. Therefore, heparin (150 units/10 ml) was used as an anticoagulant during the collection of venous blood. Since K-perchlorate also had no effect on the determination of glyoxylate as 1,5-diphenylformazan, plasma was deproteinized by mixing with one-tenth volume of 6.6 N perchloric acid, neutralized

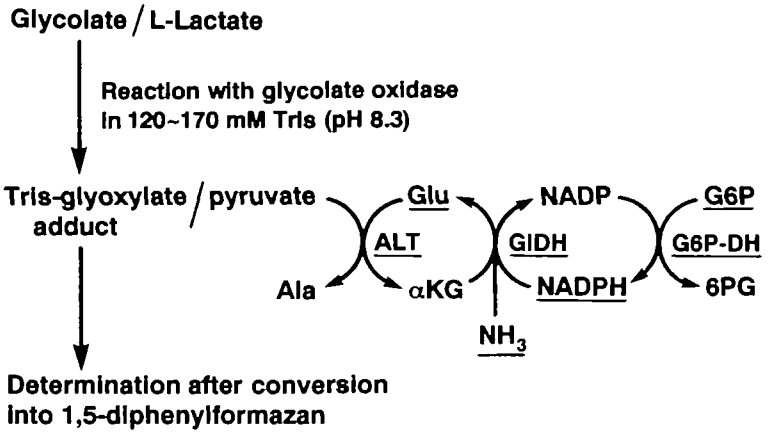

Fig. 8. **Elimination of pyruvate without affecting glyoxylate to allow the selective measurement of glycolate with glycolate oxidase.** Abbreviations used are: Ala, L-alanine; Glu, L-glutamate; α-KG, α-ketoglutarate; G6P, glucose 6-P; 6PG, 6-phosphogluconate; ALT, alanine aminotransferase; GlDH, glutamate dehydrogenase; G6P-DH, glucose 6-P dehydrogenase.

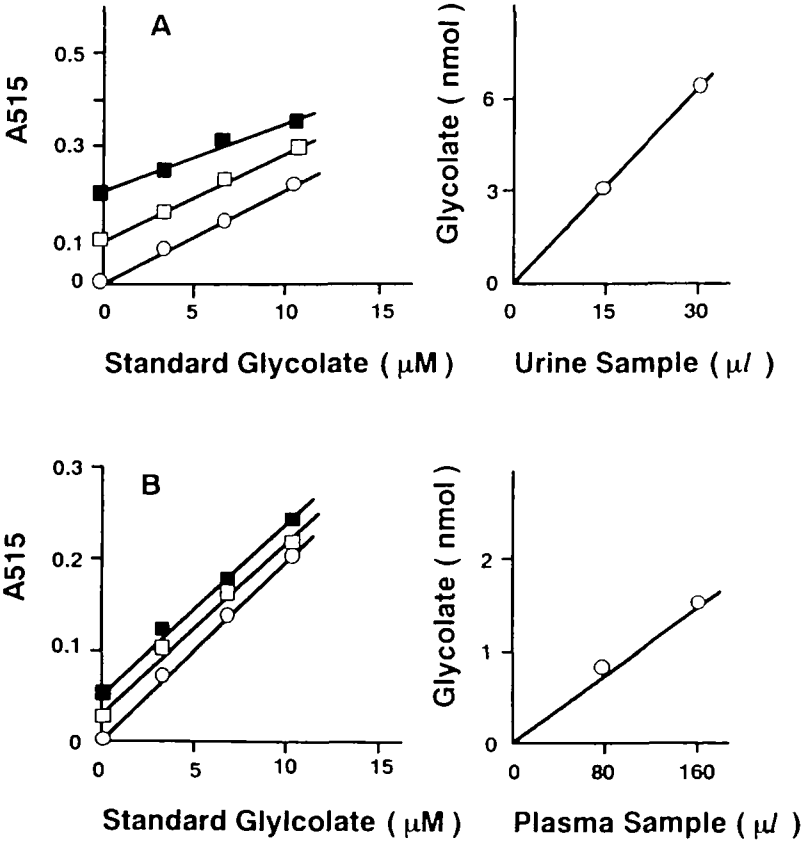

Fig. 9. **Actual data of the determination of glycolate in a urine sample (A) and a plasma sample (B).** In A, 15 μl (□) or 30 μl (■) aliquots of a urine sample were subjected to glycolate determination under the standard assay conditions shown in Fig. 2, *i.e.*, in the absence or presence of 1.5, 3.0, and 4.5 nmol of standard glycolate. ○ in the left figure: minus sample control. Each ΔA_{515} value obtained was corrected for the minus GO control. ΔA_{515} due to glycolate in the sample and a_{mM} of each glycolate determination were obtained from the intercept on the ordinate and the slope of the line, respectively. The amount of glycolate was calculated as described under "MATERIALS AND METHODS." The amounts of glycolate in the 15 and 30 μl urine samples were on or very close to a linear line as shown in the right-hand figure. In B, glycolate in a plasma sample was determined in the same way, except that 80 μl (□) and 160 μl (■) aliquots were used.

to pH 6.0–6.5 with KOH, and the K-perchlorate precipitate was removed following centrifugation in the cold. Urine was acidified with a one-hundredth volume of 6 N HCl, and neutralized to pH 6.0–6.5 with NaOH without the addition of EDTA, so that the plasma and urine samples for glycolate determination were prepared separately from those for oxalate determination (*cf.* Ref. *19*).

In our assay system, a control incubation without GO was always run simultaneously for every sample to correct the ΔA_{515} values for compounds other than glycolate (*cf.* Fig. 2), but a low control value was highly desirable. Therefore, the neutralized urine and plasma samples were incubated with one-twentieth volume of 100 mM phenylhydrazine (pH 6.0) for 15 min at room temperature, and then subjected to treatment with ~80 mg/ml of stearate-deactivated activated charcoal. The charcoal treatment effectively removed phenylhydrazine and phenylhydrazones of pyruvate and glyoxylate. Incubation of urine or plasma samples with ~5 mM phenylhydrazine prior to the charcoal treatment had no effect on the determined level of glycolate. In addition, when [^{14}C]glycolate was added to the urine before acidification with one-hundredth volume of 6 N HCl, it was recovered almost quantitatively (~97%) following the entire procedure for the preparation of charcoal-treated urine samples. Likewise, the recovery of [^{14}C]glycolate from charcoal-treated plasma samples was calculated to be ~96%, after allowing a correction factor of 5% for plasma solids. In 0.1–0.16 N HCl, however, the recovery of [^{14}C]glycolate following treatment with ~80 mg/ml of stearate-deactivated activated charcoal was only ~60%.

Dekker and Maitra (*20*) claimed that the determination of glyoxylate after conversion to 1,5-diphenylformazan may not be applicable to biological samples containing a significant concentration of Fe^{2+} or Fe^{3+}, owing to ferric ferrocyanide formation. However, the concentration of Fe in human urine and plasma are expected to be less than 10 and 36 μatoms/liter, respectively, and we obtained nearly the same ΔA_{515} when urine samples were subjected to (i) 15-min incubation at 37°C with 25 mg/ml Chelex 100, followed by treatment with stearate-deactivated charcoal, (ii) charcoal treatment, followed by 15-min incubation with Chelex 100, and (iii) charcoal treatment alone.

Determination of Glycolate in Human Urine and Plasma—Figure 9 shows actual data from the determination of glycolate in a urine sample (A) and plasma sample (B). In every determination of glycolate, at least two different amounts of sample were used, and after each the ΔA_{515} value was corrected for the corresponding "minus GO control." Plots such as those shown in this figure were prepared. The plots of ΔA_{515} *vs.* the concentration of standard glycolate were usually linear, although the slope of the linear lines representing the efficiency (a_{mM}) of each determination tended to decrease as the volume of sample was increased. The intersecting point on the ordinate axis of the standard "minus sample control" line was, in most case, at or very close to the origin of the axes. The ΔA_{515} due to glycolate in the sample was read from the intersects on the ordinate axis of the lines. Then the amount of glycolate in each sample was calculated from the ΔA_{515} readings on the ordinate axis and the a_{mM} of each determination. The calculated amounts of glycolate were usually proportional to the sample volume, provided that the amounts of sample used

TABLE I. **Repeated determination of glycolate concentration in human urine and plasma samples.** A urine sample for the determination of glycolate was prepared from the 24-h urine of a healthy woman (39 yr), and the glycolate concentration in the urine sample was determined in duplicate on 6 different days using a microplate reader as described under "MATERIALS AND METHODS". Morning samples of venous blood were taken from 6 healthy men and women (32–64 yr) under fasting conditions, and the combined plasma was subjected to deproteinization, followed by incubation with phenylhydrazine and charcoal treatment. Glycolate in the plasma sample was determined in duplicate on 4 different days. The the glycolate concentrations in the urine and plasma were calculated. Mean ± SD was calculated from the mean of the duplicate determinations on each day.

	Urine glycolate		Plasma glycolate	
			(μM)	
1	318	338	9.6	8.7
2	331	309	11.5	10.1
3	342	354	10.9	11.7
4	370	374	11.5	11.5
5	369	414		
6	387	324		
Mean ± SD.	353 ± 27		10.7 ± 1.0	

TABLE II. **Urinary excretion of glycolate and oxalate by 12 inpatients receiving treatments for conditions unrelated to urinary tract stone formation.** Body surface area was calculated from height and body weight using a monograph. Glycolate was determined using a microplate reader as described under "MATERIALS AND METHODS." Oxalate and creatinine were determined by a spectrophotometric method with oxalate oxidase (*19*) and Jaffe's reaction, respectively.

Subjects	Age	Sex	Body weight (kg)	Height (cm)	Creatinine (μmol/d)	Glycolate		Glycolate/creatinine ratio	Oxalate		Oxalate/creatinine ratio
						(μmol/d)	(μmol/d/1.73 m²)		(μmol/d)	(μmol/d/1.73 m²)	
1	46	M	67.3	166	7,140	255	252	0.036	199	197	0.028
2	58	M	46.1	163	2,090	75	90	0.036	86	103	0.041
3	62	M	44.8	162	4,130	183	221	0.044	274	331	0.063
4	68	M	52.8	167	4,370	99	109	0.023	91	100	0.021
5	54	M	59.5	170	9,570	301	312	0.032	293	304	0.031
6	46	M	72.8	174	11,570	271	251	0.023	176	163	0.015
7	51	F	49.5	159	8,820	277	323	0.031	166	194	0.019
8	61	F	64.6	149	4,470	105	115	0.024	170	186	0.038
9	45	F	29.5	159	5,580	160	235	0.029	225	330	0.040
10	50	F	46.5	157	4,690	170	205	0.036	151	183	0.032
11	54	F	50.0	155	3,860	240	285	0.062	117	139	0.030
12	43	F	56.7	152	10,100	508	575	0.052	484	547	0.048
mean ± SD					6,370 ± 2,990	220 ± 118	248 ± 127	0.036 ± 0.012	203 ± 110	231 ± 127	0.034 ± 0.014

were adequate, so that all ΔA_{515} readings were made between 0.02 and 0.5 and the a_{mM} of each determination was above 13.

In the experiment shown in Table I, the precision of the proposed method was tested by analyzing glycolate in a urine sample in duplicate 6 times and in a plasma sample in duplicate 4 times on different days. When the mean of the duplicate determinations was taken as the glycolate concentration determined on each day, the mean ± SD and inter-run C.V. for urinary glycolate were calculated to be 353 ± 27 μM and 7.6%, while those for plasma glycolate were 10.7 ± 1.0 mM and 9.3%, respectively. Data for urinary glycolate and oxalate excretion of 12 inpatients in our university hospital are presented in Table II. None of these patients was suffering from urinary tract stone formation or kidney diseases. The daily urinary excretion of glycolate and the glycolate/creatinine ratio determined by the proposed spectrophotometric method were 248 ± 127 μmol/1.73 m^2 of body surface area and 0.036 ± 0.012 (mean ± SD), respectively, whereas the oxalate excretion and oxalate/creatinine ratio were 231 ± 127 μmol/1.73 m^2 of body surface area and 0.034 ± 0.014, respectively. The oxalate excretion may have been slightly overestimated because the 24-h urine used was collected from inpatients in the absence of HCl, but nevertheless the urinary glycolate excretion was determined to be slightly more than that of oxalate. Table III shows the plasma glycolate level of 11 healthy subjects determined by the proposed method. The mean value of 7.9 μM obtained was very close to the 8.0 μM (*13*) and 7.9 μM (*6*) values recently determined by a method involving the enzymatic conversion of glycolate into glyoxylate and high-performance liquid chromatographic separation of phenylhydrazone of glyoxylate formed. In one experiment, the concentration of glycolate in the serum was measured to be higher (15.7 μM) than that in plasma, but since the isolation of plasma is simpler than that of serum and the glycolate concentration in 24-h urine and plasma reflects the *in vivo* metabolism of glycolate, glyoxylate and oxalate, we did not investigate further the determination of glycolate in serum.

One disadvantage of this spectrophotometric method is that a small deviation in the a_{mM} of each glycolate determination significantly influenced the calculated glycolate concentration, because in the calculation ΔA_{515} or ΔA_{520} is divided by a_{mM} (see "MATERIALS AND METHODS"). For example, when the ΔA_{515} due to glycolate in 30 μl of a urine sample is 0.15 the glycolate concentrations in the sample using a_{mM} values of 15 and 16 are calculated to be 187 and 175 μM, respectively. Therefore, the ΔA_{515} *vs.* concentration of standard glycolate plots should be on or at least very close to a linear line, and empirically, a_{mM} determined with a microplate reader should not be below 13. Fortunately, however, re-examination of the glycolate concentration was possible when the results obtained were unsatisfactory, because we did not notice, unlike in the case of oxalate determination, any increase in the glycolate level in neutralized urine and plasma samples upon freezing and thawing. Based on the results presented above, we believe that the proposed spectrophotometric method for the determination of glycolate in urine and plasma is simple yet fairly reliable, and proudes a valuable tool for the differential diagnosis of hyperoxaluria syndromes and in studies of glyoxylate metabolism.

TABLE III. **Determination of glycolate concentration in human plasma.** Morning samples of venous blood were taken before breakfast from 11 healthy volunteers in the presence of 150 units/10 ml heparin, and the plasma obtained was stored frozen at −80°C. The glycolate concentration of each plasma was determined with a microplate reader as described under "MATERIALS AND METHODS" using 80 and 160 μl plasma samples.

Subject	Age	Sex	Plasma glycolate (μM)
1	20	M	10.5
2	23	M	9.9
3	22	M	6.9
4	21	M	9.4
5	21	M	9.2
6	19	M	7.3
7	19	M	5.2
8	22	M	8.0
9	24	M	7.0
10	21	M	6.2
11	29	F	7.4
Mean ± S.D			7.9 ± 1.7

We thank Prof. Takashi Kanno, Department of Clinical Inspection Medicine of our medical school, for allowing us to determine the glycolate and oxalate concentrations in 24-h urine of 12 inpatients in our university hospital. We are indebted to the medical students of our school for their generous donation of morning samples of venous blood.

REFERENCES

1. Xue, H.-H., Fujie, M., Sakaguchi, T., Oda, T., Ogawa, H., Kneer, N.M., Lardy, H.A., and Ichiyama, A. (1999) Flux of the L-serine metabolism in rat liver. The predominant contribution of serine dehydratase. *J. Biol. Chem.* **274**, 16020–16027
2. Xue, H.-H., Sakaguchi, T., Fujie, M., Ogawa, H., and Ichiyama, A. (1999) Flux of the L-serine metabolism in rabbit, human, and dog livers. Substantial contributions of both mitochondrial and peroxisomal serine:pyruvate/alanine:glyoxylate aminotransferase. *J. Biol. Chem.* **274**, 16028–16033
3. Harris, K.S. and Richardson, K.E. (1980) Glycolate in the diet and its conversion to urinary oxalate in the rat. *Invest. Urol.* **18**, 106–109
4. Poore, R.E., Hurst, C.H., Assimos, D.G., and Holmes, R.P. (1997) Pathways of hepatic oxalate synthesis and their regulation. *Am. J. Physiol.* **272**, C289–294
5. Danpure, C.J. and Purdue, P.E. (1995) Primary hyperoxaluria in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds.) Vol. II, pp. 2385–2424, McGraw-Hill, New York
6. Marangella, M., Petrarulo, M., Vitale, C., Cosseddu, D., and Linari, F. (1992) Plasma and urine glycolate assays for differentiating the hyperoxaluria syndromes. *J. Urol.* **148**, 986–989
7. Calkins, V.P. (1943) Microdetermination of glycolic and oxalic acids. *Anal. Chem.* **15**, 762–763
8. Dagley, S. and Rodgers, A. (1953) Estimation of glycollic acid. *Biochim. Biophys. Acta* **12**, 591
9. Viccaro, J.P. and Ambye, E.L. (1972) Colorimetric determination of glycolic acid with β-naphthol. *Microchem. J.* **17**, 710–718
10. Hockaday, T.D.R., Frederick, E.W., Clayton, J.E., and Smith, L.H., Jr. (1965) Studies on primary hyperoxaluria II. Urinary oxalate, glycolate, and glyoxylate measurement by isotope dilution methods. *J. Lab. Clin. Med.* **65**, 677–687
11. Chalmers, R.A. and Watts, R.W.E. (1972) The quantitative extraction and gas-liquid chromatographic determination of organic acids in urine. *Analyst* **97**, 958–967
12. Petrarulo, M., Pellegrino, S., Bianco, O., Marangella, M., Linari, F., and Mentasti, E. (1989) Derivatization and high-performance liquid chromatographic determination of urinary glyco-

lic acid. *J. Chromatogr.* **465**, 87–93
13. Petrarulo, M., Marangella, M., and Linari, F. (1991) High-performance liquid chromatographic determination of plasma glycolic acid in healthy subjects and in cases of hyperoxaluria syndromes. *Clin. Chim. Acta* **196**, 17–26
14. Kasidas, G.P. and Rose, G.A. (1979) A new enzymatic method for the determination of glycollate in urine and plasma. *Clin. Chim. Acta* **96**, 25–36
15. Metzger, R.P., Sauerheber, R.D., Lyons, S.A., and Westall, J.R. (1975) The effect of streptozotocin diabetes on the levels of glycolate and lactate excreted in rat urine. *Arch. Biochem. Biophys.* **169**, 555–559
16. Tokushige, M. and Sizer, I.W. (1967) Glycolate oxidase of hog kidney. *J. Biochem.* **62**, 719–725
17. Matsui, M., Okada, M., and Ishidate, M. (1965) Structure of the principal colored product formed in the determination of glycolic acid. *Anal. Biochem.* **12**, 143–149
18. McFadden, B.A. and Howes, W.V. (1960) The determination of glycolic acid in biological systems. *Anal. Biochem.* **1**, 240–248
19. Ichiyama, A., Nakai, E., Funai, T., Oda, T., and Katafuchi, R. (1985) Spectrophotometric determination of oxalate in urine and plasma with oxalate oxidase. *J. Biochem.* **98**, 1375–1385
20. Dekker, E.E. and Maitra, U. (1962) Conversion of γ-hydroxyglutamate to glyoxylate and alanine; Purification and properties of the enzyme system. *J. Biol. Chem.* **237**, 2218–2227