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Origin of D-serine present in urine of mutant mice lacking D-amino-acid oxidase activity

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Summary. Urine of ddY/DAO$^-$ mice lacking D-amino-acid oxidase contained 5.7 times more serine than that of normal ddY/DAO$^+$ mice. Most of the serine was D-isomer. The origin of this D-serine was examined. Oral administration of 0.02% amoxicillin and 0.004% minocycline to the ddY/DAO$^-$ mice for 7 days did not reduce the urinary D-serine, indicating that the D-serine was not of intestinal bacterial origin. When the mouse diet was changed to one with different compositions, the urinary D-serine was considerably reduced. Furthermore, starvation of the ddY/DAO$^-$ mice for 24 hours reduced the urinary D-serine to 33% of the original level. These results indicate that most of the urinary D-serine comes from the diet. However, the urine of the starved ddY/DAO$^-$ mice still contained 4.6 times more D-serine than that of the ddY/DAO$^+$ mice, suggesting a part of the D-serine have an endogenous origin.

Keywords: D-Serine – Urine – Mouse – D-Amino-acid oxidase – D-Amino acids

Introduction

D-Amino-acid oxidase (EC 1.4.3.3) catalyzes oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding 2-oxo acids, producing hydrogen peroxide and ammonia in the course of the reaction (Krebs, 1935). This enzyme is present in the kidneys, livers, and brains of higher animals (Meister, 1965). The physiological role of this enzyme has long been unclear because its substrates, D-amino acids, are considered to be very rare in higher animals (Meister, 1965; Konno and Yasumura, 1992).

It has been believed for a long time that amino acids are exclusively L-isomers in eukaryotes, especially in higher animals (Corrigan, 1969). However, developments and improvements of analytical methods to separate D-amino acids have revealed the presence of various D-amino acids in a variety

of organisms. Specific functions have been proposed for some D-amino acids. D-Aspartic acid was detected in embryos and neonates of chickens and rodents, and is suggested to play a role in early development (Dunlop et al., 1986; Neidle and Dunlop, 1990). D-Serine is abundant in the brains of mammals and is suggested to potentiate the activity of the *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors of neurons (Hashimoto et al., 1993a; Matsui et al., 1995; Schell et al., 1995).

For the study of a physiological function of a specific enzyme, mutant animals having disorders in the enzyme are useful. We have established a mutant mouse strain (ddY/DAO$^-$) lacking D-amino-acid oxidase activity (Konno and Yasumura, 1983, 1984). Various D-amino acids were detected at high concentrations in the organs, serum, and urine of the ddY/DAO$^-$ mice. D-Methionine and D-alanine present in the urine were determined to come from their diet and intestinal bacteria, respectively (Konno et al., 1988, 1990, 1993). Therefore, one of the physiological functions of D-amino-acid oxidase has been assigned to the metabolism of D-amino acids derived from intestinal bacteria. D-Serine was also detected in the urine and organs of the ddY/DAO$^-$ mice (Konno et al., 1991; Nagata et al., 1992; Hashimoto et al., 1993b) but its origin has not been clear. Therefore, this problem was examined.

Materials and methods

Mice

Normal ddY/DAO$^+$ mice and mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity were used. They were raised on the stock NMF diet (Oriental Yeast, Tokyo) and maintained at 24 ± 2°C with a 12-hour light/dark cycle (light, 8:00–20:00; dark, 20:00–8:00). Adult male mice were used for experiments.

Urine collection

Mice were individually put into metabolic cages at 17:00 and their urine was collected continuously during the following 17 hours. During the time they were given the diet and water ad libitum.

Antibiotics administration

Five ddY/DAO$^-$ mice were housed in a cage with a mesh floor to prevent access to their feces. They were given ad libitum the NMF diet and water containing both 0.02% amoxicillin (an antibiotic which inhibits the synthesis of bacterial cell walls, Pasetocin®, Kyowa Hakko Kogyo, Tokyo) and 0.004% minocycline (an antibiotic which inhibits bacterial protein synthesis, Minomycin®, Lederle (Japan), Tokyo) for 7 days. During this period, their cage and water containing the antibiotics were changed every other day. On the 7th day, the mice were transferred to metabolic cages for urine collection.

Effect of the diet on urinary D-serine

A modified AIN-76M diet was made for us by a commercial company (CLEA Japan, Tokyo). The original AIN-76 formula contains 0.3% DL-methionine as a supplement (American Institution of Nutrition, 1977). To avoid any effect of the D-methionine, 0.3% DL-methionine was replaced by 0.3% L-methionine in the AIN-76M diet. Other components in the AIN-76M diet were the same as those in the original AIN-76 diet.

The ddY/DAO$^-$ mice which had been maintained on the NMF diet were fed the modified AIN-76M diet for 7 days. On the last day, they were transferred to metabolic cages for urine collection.

Starvation

The five ddY/DAO$^-$ mice which had been maintained on the NMF diet were transferred to a cage with a mesh floor at 17:00. For the following 24 hours, they were not given the diet but were given 5% sucrose solution and water ad libitum. The 5% sucrose solution was given, otherwise they excreted a very small quantity of urine. At 17:00, they were individually put into metabolic cages and their urine was collected during the following 17 hours. During this period, they were given 5% sucrose solution and water ad libitum.

Amino acid analysis of urine

To about 1 ml of the mouse urine was added distilled water to make up 4 ml and 20 μl of 10 mM α-amino-β-guanidino propionic acid (AGPA, an internal standard to check the recovery of the amino acids). This solution was mixed with an equal volume of 10% trichloroacetic acid (TCA) and was kept at 4°C overnight. Since a large volume of urine was obtained from the starved mice, 20 μl of 10 mM AGPA and 1/10 volume of 50% TCA were added to the whole urine (about 10 ml) and kept at 4°C overnight. The mixture was centrifuged at 8,000 × g for 7 min. The supernatant solution was applied to a Dowex 50W column (1.8 cm × 4 cm, Muromachi Kagaku Kogyo, Tokyo). The resin was washed with distilled water to remove unbound materials and TCA. The bound amino acids were eluted with about 40 ml of 3 M ammonium hydroxide. The eluate was dried under reduced pressure. The dried residue was dissolved in 0.7 ml distilled water. A part of this solution (about 70 μl) was mixed with an equal volume of 0.04 N HCl, passed through a 0.2 μm filter (Ultrafree C3GV, Japan Millipore, Tokyo), and examined for amino acid concentrations using an amino acid analyzer (Model L8500, Hitachi, Tokyo).

Creatinine content in the urine was determined using an assay kit (Wako Pure Chemical Industries, Osaka). Amino acid concentrations in the urine were expressed on the basis of its creatinine content.

Separation and quantification of D- *and* L-*serine*

The rest of the amino acid solution above (about 600 μl) was streaked on a thin-layer plate (LK6, Whatman, Clifton) and developed with l-butanol : acetic acid : water (3 : 1 : 1, V/V). After the plate was dried, 0.2% ninhydrin solution was sprayed on both edges of the plate where standard L-serine had been applied. A zone corresponding to the standard L-serine was scraped off and was mixed with 0.7 ml of distilled water. The slurry was passed through a 0.2 μm filter (Corning Class Works, Corning).

The isomer composition of urinary serine was determined according to the method of Marfey (1984) with a slight modification as described before (Konno et al., 1989). The above filtrate (60 μl) was mixed with 180 μl of 0.05% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, known as Marfey's reagent, Pierce, Rockford) in acetone. Forty μl of 1 M sodium bicarbonate was added and the solution was incubated at 40°C for 1 hour. After cooling to room temperature, 20 μl of 2 N HCl was added, and 10 μl of the solution was applied to a reversed-phase column (4.6 mm × 150 mm, Puresil 5C18, Waters, Milford). An HPLC system (Model 600E pump, Model 600 System Controller, Model 486 Tunable Absorbance Detector, and M741 Data Module, Waters) was used to separate and quantify D- and L-serine derivatives. A mobile phase consisting of solution A (0.05 M triethylamine phosphate, pH 3.1) and solution B (acetonitrile) was used for elution. Linear gradients were programmed as follows: 0–45 min, 90 : 10 (A : B)–65:35; 45–46 min, 65 : 35–0 : 100; 46–55 min, 0 : 100; 55–56 min, 0 : 100–90 : 10. The flow rate was 1.5 ml/min and the detector was set at 340 nm.

The concentrations of the D-serine and L-serine in the urine were determined by multiplying the serine concentration obtained in the amino acid analysis with the ratio of peak area of the D-serine derivative to that of the L-serine derivative.

Statistical analysis

The significance of the difference between the mean values of amino acid concentrations obtained before and after the various treatments was determined by the *t*-test.

Results

Amino acid concentrations in urine were examined in the normal ddY/DAO$^+$ mice and the mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase. Figure 1 shows that urine of the ddY/DAO$^-$ mice contained several amino acids in higher concentrations than that of the ddY/DAO$^+$ mice. The conspicuous amino acids were alanine (20.7 times) and methionine (19.7 times). Proline (6.3 times), serine (5.7 times), threonine (2.5 times), leucine (2.4 times), tyrosine (2.2 times), and lysine (2.1 times) were also abundant. The reason why large amounts of alanine and methionine are present in their urine has been clarified (Konno et al., 1988, 1990, 1993). Since D-serine has recently been suggested to have an important role in the mammals (Hashimoto et al.,

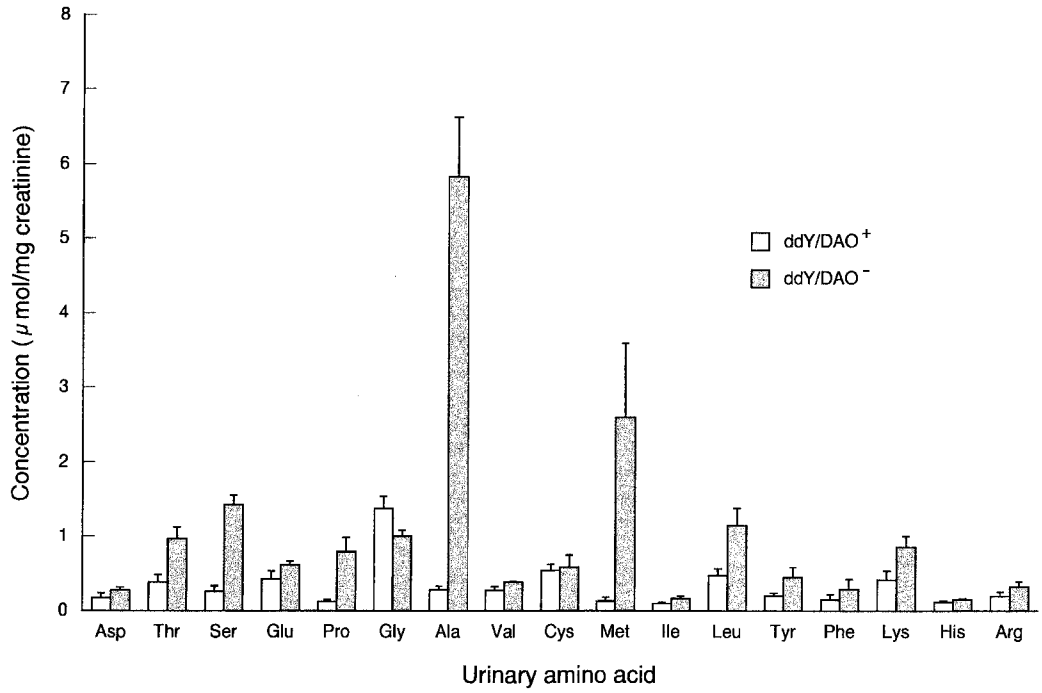

Fig. 1. Amino acid concentrations in mouse urine. Amino acids were purified from the urine of normal ddY/DAO$^+$ and mutant ddY/DAO$^-$ mice by deproteinization and cation-exchange chromatography. Their concentrations were determined using an amino acid analyzer. Bars show standard deviation

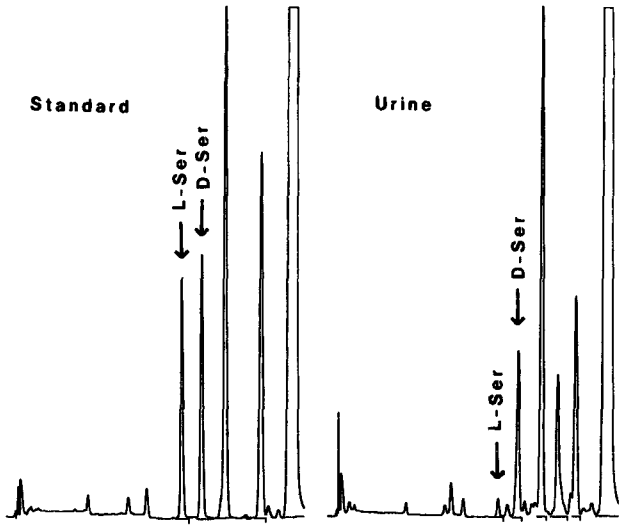

Fig. 2. Analysis of isomer composition of urinary serine. Serine was purified from urine of a ddY/DAO$^-$ mouse lacking D-amino-acid oxidase by deproteinization, cation-exchange chromatography, and thin-layer chromatography. It was derivatized with FDAA and the resultant D- and L-serine derivatives were separated on a reversed-phase column by high performance liquid chromatography. Left panel: standard DL-serine. Right panel: serine purified from the urine of a ddY/DAO$^-$ mouse

1993a; Schell et al., 1995), we examined whether D-serine was present in the urine of the ddY/DAO$^-$ mice.

Amino acids were purified from the urine of the ddY/DAO$^-$ mice. Serine was separated from other amino acids. It was derivatized with FDAA and the resulting D- and L-serine derivatives were separated on a reversed-phase column. Figure 2 shows that most of the urinary serine was D-isomer. The amount of the D-serine was 8.4 times more than that of L-serine on the average of six ddY/DAO$^-$ mice. D-Serine was also detected in the urine of the normal ddY/DAO$^+$ mice but its quantity was quite low (about 1/14 of the amount present in the ddY/DAO$^-$ mice).

The origin of the urinary D-serine was examined. First, the effects of intestinal bacteria on the D-serine were examined. The ddY/DAO$^-$ mice were given water containing both 0.02% amoxicillin and 0.004% minocycline for 7 days. Figure 3 shows that the antibiotic administration drastically reduced the urinary alanine content. This is because most of the alanine is D-alanine derived from intestinal bacteria (Konno et al., 1993). This result, therefore, indicates that most of intestinal bacteria were eliminated from the ddY/DAO$^-$ mice by the antibiotic treatment. However, the serine content was not changed (Fig. 3), and the D-serine content was not changed either (Fig. 4). These results indicate that the D-serine present in the urine of the ddY/DAO$^-$ mice is not of bacterial origin.

Next examined was whether the urinary D-serine was of dietary origin. The ddY/DAO$^-$ mice were usually fed the NMF diet. This diet is a closed formula diet which does not show its exact composition. Then, an open formula AIN-

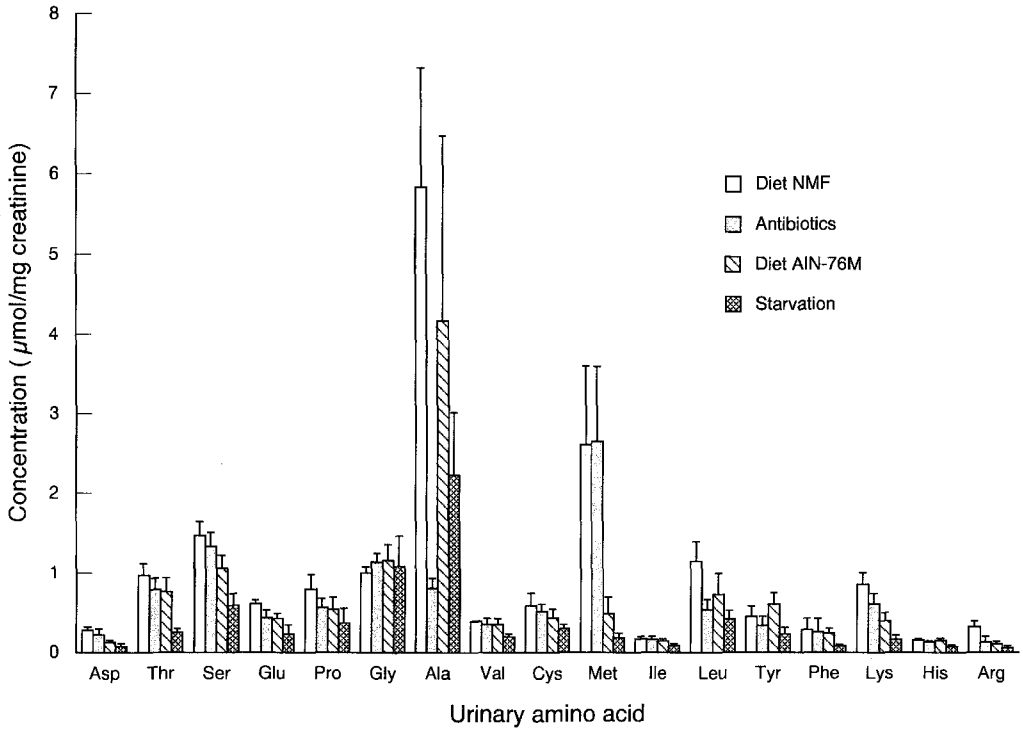

Fig. 3. Effects of antibiotics and feedings on the urinary amino acid concentrations. The ddY/DAO$^-$ mice were raised and maintained on the stock diet (column: diet NMF). The first group of these mice was given water containing both 0.02% amoxicillin and 0.004% minocycline for 7 days to eliminate intestinal bacteria (column: antibiotics). The second group was fed for 7 days a different diet which had different compositions and did not contain supplementary DL-methionine (column: diet AIN-76M). The third group was not given the diet for 24 hours (column: starvation). Following the various treatments, their urine was collected for 17 hours and analyzed for amino acid concentrations. Bars show standard deviation

76 diet was chosen for this experiment. However, this diet is formulated to contain 0.3% DL-methionine as a supplement (American Institution of Nutrition, 1977). To avoid any possibility of the supplementary D-methionine affecting the urinary D-serine, a modified diet was made in which 0.3% DL-methionine was replaced with 0.3% L-methionine. This diet was named an AIN-76M diet. When the ddY/DAO$^-$ mice were fed this AIN-76M diet for 7 days, the urinary methionine content was drastically reduced (Fig. 3). This was because most of the methionine in the urine of the ddY/DAO$^-$ mice on the NMF diet was D-methionine derived from the D-methionine supplement in this diet (Konno et al., 1988). Figure 3 also shows that the ddY/DAO$^-$ mice fed the AIN-76M diet excreted less serine into their urine than their counterparts on the NMF diet. D-Serine content was also reduced to 68% of the original level (Fig. 4). The difference was statistically significant ($P < 0.01$). Therefore, these results suggest that some part of the urinary D-serine came from the diet.

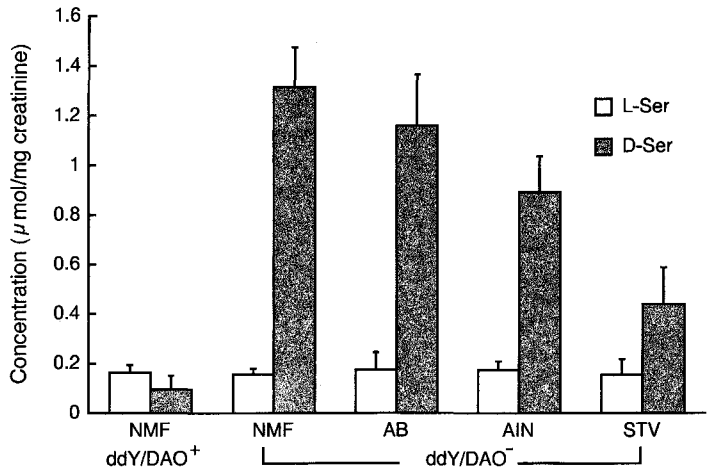

Fig. 4. Effects of antibiotics and feedings on the D- and L-serine concentrations in urine. The normal ddY/DAO$^+$ mice and mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase were raised and maintained on the stock diet (label: NMF). The first group of these ddY/DAO$^-$ mice was given water containing both 0.02% amoxicillin and 0.004% minocycline for 7 days to eliminate intestinal bacteria (label: AB). The second group was fed for 7 days a different diet which had different compositions and did not contain supplementary DL-methionine (label: AIN). The third group was not given the diet for 24 hours (label: STV). Following the various treatments, their urine was collected for 17 hours and analyzed for D- and L-serine concentrations. Bars show standard deviation

To further examine the possibility that the D-serine comes from the diet, the ddY/DAO$^-$ mice were not given the diet for 24 hours and their urine was collected during the subsequent 17 hours. In this case, the urinary serine content and the D-serine content was reduced to 44% and 33% of the original level, respectively (Figs. 3 and 4). These differences were statistically significant ($P < 0.01$). Therefore, these results confirm that most of the D-serine present in the urine of the ddY/DAO$^-$ mice comes from the diet. However, Fig. 4 also shows that the starved ddY/DAO$^-$ mice still excreted 4.6 times more D-serine in their urine than the normal ddY/DAO$^+$ mice. Therefore, not all the D-serine in the urine came from the diet. Part of the D-serine most likely had an endogenous origin.

Discussion

Since an administration of the antibiotics to the ddY/DAO$^-$ mice did not reduce the urinary D-serine in contrast to the alanine (Figs. 3 and 4), the D-serine was considered to be not of bacterial origin. This conclusion is consistent with the previous reports. D-Serine is not a component of the bacterial cell wall though D-alanine and D-glutamic acid are important constituents (Wheat, 1988). When the ddY/DAO$^-$ mice were made germ-free at birth and maintained in a germ-free environment, the urinary D-alanine was reduced to a very low level (Konno et al., 1993) but the urinary serine was not reduced

(unpublished data). Therefore, it is unlikely that the D-serine in the urine comes from intestinal bacteria.

It was concluded that most of the D-serine present in the urine of the ddY/DAO$^-$ mice came from the diet they were fed. The NMF diet is a closed formula diet. Our amino acid analysis showed that a substantial amount of free serine was present in this diet. However, free D-serine was not detected in this diet (data not shown). The AIN-76 diet is an open formula diet; it should not contain free serine. Indeed, our analysis confirmed that the AIN-76M diet did not contain free D-serine or L-serine (data not shown). It was not determined, however, how much protein-bound D-serine was present in these diets. It is difficult to accurately quantify the protein-bound D-amino acids. Before analysis, proteins in the diet have to be hydrolyzed. However, hydrolysis of proteins in 6N HCl at 110°C for 24 hours inevitably causes racemization of amino acids (Liardon et al., 1981). The rate of racemization of each amino acid changes depending on the type of proteins and the positions of the amino acids in the peptides (Liardon et al., 1981; Manning, 1970; Toyo'oka and Liu, 1995). Even under milder conditions of hydrolysis using a lower temperature and a shorter exposure time, racemization of amino acids occurs (D'Aniello et al., 1990). Therefore, an accurate determination of protein-bound D-amino acids has to wait for the development of new hydrolysis methods. However, it is probable that both diets contained protein-bound D-serine, because heating processes are involved in the production of diets in the factories and the heating of proteins is known to cause racemization of the constituent amino acids. Serine is known to be one of the amino acids which are prone to racemization during heating (Man and Bada, 1987).

To examine the effects of the diet on the urinary D-serine, the ddY/DAO$^-$ mice were starved for 24 hours and their urine was collected during the subsequent 17 hours. It is a question that the 24-hour starvation is enough to deplete the amino acids stored inside the tissues of the animals. This period of starvation may not be enough. However, since the urinary methionine level of the ddY/DAO$^-$ mice was reduced to the level of normal mice after the 24-hour starvation (Fig. 3), it seems that a considerable amount of D-methionine which had been ingested from the NMF diet and was present inside the tissues of the ddY/DAO$^-$ mice was excreted into their urine during the 24-hour period of starvation. Similarly, a large part of the D-serine taken up from the diet would be excreted into the urine during this period. Starvation over 24 hours would not be conducive to safe animal welfare.

Figure 4 shows that the normal ddY/DAO$^+$ and mutant ddY/DAO$^-$ mice excreted a similar level of L-serine into their urine. It also shows that the excretion of L-serine into the urine was constant in the ddY/DAO$^-$ mice even when the excretion of D-serine was changed. Therefore, there seems to be a strict recognition of stereospecificity of amino acids for reabsorption in the proximal tubules of the kidney. Indeed, Kragh-Hansen and Sheikh (1984) have shown that there are multiple transport systems for L-serine but only one system for D-serine in the proximal tubules of the rabbit kidney. A transport system in the pars convoluta of the proximal tubule transports both L- and D-serine but has a higher affinity for L-serine than D-serine.

Therefore, most D-amino acids would not be reabsorbed in the proximal tubules and excreted into the urine. D-Amino-acid oxidase is present in the proximal tubules and therefore may function to metabolize the reabsorbed D-amino acids to keep D-amino acids at a low level in the body.

D-Serine has been shown to be present in higher animals. Brückner and Haush (1990) found that D-serine was present in human blood. Nagata et al. (1992) showed that D-serine was present in the serum and tissues of both ddY/DAO$^+$ and ddY/DAO$^-$ mice. Hashimoto et al. (1992) and Nagata (1992) showed the presence of D-serine in rodent brains. These findings were verified by other investigators not only in rodents but also in other animals including humans (Hashimoto et al., 1993a; Brückner and Hausch, 1993; Nagata et al., 1994; Fukushima et al., 1995; Goodnough et al., 1995; Schell et al., 1995). D-Serine is considered to potentiate the function of the NMDA receptors of neurons by binding to the glycine modulatory sites (Matsui et al., 1995; Schell et al., 1995). Therefore, there is a high possibility that D-serine is synthesized in higher animals, though clear evidence has not yet been obtained. Schell et al. (1995) consider the possibility that D-serine biosynthesis involves the glycine cleavage pathway. Other mechanisms involving rasemase and transaminase are also possible. Our results are in line with the biosynthesis of D-serine. Since such D-serine is not metabolized in the mutant ddY/DAO$^-$ mice due to the lack of D-amino-acid oxidase, it is finally excreted and accumulates in the urine as observed in the present study. The ddY/DAO$^-$ mice would be useful for the exploration of the biosynthesis pathway for D-amino acids because they do not have a potent catabolic pathway for D-amino acids.

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