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D-Aminoaciduria in mutant mice lacking D-amino-acid oxidase activity

R. Konno[1], M. Ikeda[2], A. Niwa[1], and Y. Yasumura[1]

Department of [1] Microbiology and [2] Laboratory of Medical Sciences,
Dokkyo University School of Medicine, Mibu, Tochigi, Japan

Summary. Urine of mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity contained more serine and proline than that of normal ddY/DAO$^+$ mice. D-Amino-acid oxidase treatment of urinary amino acids decreased the serine and proline, suggesting that they contained D-isomers. An HPLC analysis confirmed the presence of D-serine. Urinary serine and proline contents were not decreased when the ddY/DAO$^-$ mice were fed a diet which did not contain supplementary D-methionine or when they were given water containing antibiotics. These results suggest that the D-serine and D-proline do not derive from the D-methionine supplemented in the diet or from intestinal bacteria. In urine of the ddY/DAO$^-$ mice, a substance which seemed to be D-methionine sulfoxide and/or D-methionine sulfone was present. It is probably a metabolite of the D-methionine supplemented in the diet. The D-aminoaciduria in the mutant mice lacking D-amino-acid oxidase activity indicates that this enzyme is involved in the metabolism of the D-amino acids in normal mice.

Keywords: Amino acids – D-Amino-acid oxidase – D-Aminoaciduria – Physiological role – Mutant mice – D-Amino acids – Urine

Introduction

D-Amino-acid oxidase (EC 1. 4. 3. 3) is a curious enzyme. It catalizes oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding 2-oxo acids with concomitant production of hydrogen peroxide and ammonia (Krebs, 1935). This enzyme is present in various organisms. Higher animals have it mainly in the kidney, liver and brain (Meister, 1965). However, its physiological role is not known, because its substrates, D-amino acids, are rare in animals. It is an interesting question why organisms have such a curious enzyme. Meister et al. (1960) documented the possibility that this enzyme has a natural substrate other than D-amino acids. Hamilton and his collaborators (1979, 1985) have proposed that thiazolidine-2-carboxylate, an adduct of glyoxylate and cysteamine, is the natural substrate. Although this hypothesis is interesting, there is little experimental evidence to support it.

Mutant organisms are useful for the elucidation of physiological function of enzymes. The role of a specific enzyme has often been determined by comparing the physiology of normal organisms with that of mutant organisms which carry disorders in the enzyme. We have established a mutant mouse strain (ddY/DAO$^-$) lacking D-amino-acid oxidase activity (Konno and Yasumura, 1983, 1984, 1988) and have been examining changes in the physiology of the ddY/DAO$^-$ mice.

Materials and methods

Mice and diet

Adult male mice of normal ddY/DAO$^+$ and mutant ddY/DAO$^-$ strains were used. They were housed in polycarbonate cages with bedding of wood shavings. Their room was maintained at 24 ± 2°C with a 12-hour light/dark cycle.

Mice were raised on a stock diet (Type NMF, Oriental Yeast Co., Tokyo, Japan). This diet contained about 0.04% DL-methionine as a supplement (Konno et al., 1988b). To examine the effects of diets on urinary amino acids, we fed the ddY/DAO$^-$ mice Rodent Laboratory Chow 5001 (an estimated value of D-methionine content was 0.0037%, Ralston Purina Co., St. Louis, U.S.A.) or a modified NMF diet (Oriental Yeast Co.) in which supplementary DL-methionine in the NMF diet was replaced with an equal amount of L-methionine (Konno et al., 1988b). The mice were maintained for at least two weeks on the new diets before urine collection.

Urine collection

Mice were individually kept in metabolic cages and urine was collected overnight (17:00–10:00). During this period, they were given the diet and water *ad libitum*.

Amino acid analysis

Amino acid concentrations in urine were determined according to the previously described procedure (Konno et al., 1988a). Briefly, urine was deproteinized with trichloroacetic acid and applied to a cation-exchange column. Bound amino acids were eluted with ammonium hydroxide and the eluate was evaporated under reduced pressure. The dried residue was dissolved in 0.2 N citrate buffer and applied to amino acid analyzers (Models KLA-5, 835 and L8500, Hitachi, Tokyo, Japan).

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

Analysis of the isomer composition of urinary serine

The isomer composition of serine in urine was determined according to the previously described procedure (Konno et al., 1988b). Briefly, amino acids were purified from urine of ddY/DAO$^-$ mice by deproteinization, cation- and anion-exchange chromatography. Serine was separated from other amino acids on a TLC plate and was extracted. The isomer composition of the serine was determined using a high performance liquid chromatograph equipped with a chiral column (Crownpak, Daicel Chemical Industries, Tokyo, Japan).

D-Amino-acid oxidase treatment of urinary amino acids

Urinary amino acids were purified by deproteinization and cation-exchange chromatography as described above. The eluate was dried under reduced pressure and the residue was dissolved in 2 ml of 50 mM pyrophosphate buffer (pH 8.3). After being filtrated through a 0.2 μm membrane, 0.8 ml of the solution was mixed with 0.2 ml of 0.1 mM FAD and 5 μl

of a crystalline suspension of hog kidney D-amino-acid oxidase (Sigma Chemical Co., St. Louis, MO, U.S.A.). A control tube did not include the oxidase suspension. These solutions were incubated at 37°C for 3 hr. The reaction was terminated by the addition of 7 ml of 5.7% trichloroacetic acid solution. The mixture was processed according to the procedure for the amino acid analysis as described above, and was measured for amino acid concentrations using the amino acid analyzer.

Administration of antibiotics

The ddY/DAO$^-$ mice were given, *ad libitum*, the stock diet and water containing 0.01% amoxicillin and 0.002% minocycline for a week. The water and their cages were changed every other day. On day 7, they were transferred to individual metabolic cages and urine was collected overnight, during which time they had free access to food and water containing the antibiotics. Their urine was measured for amino acid concentrations as described above.

Results and discussion

Amino acid analyses showed that urine of mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity contained more than 10-fold alanine and 3-fold methionine than that of normal ddY/DAO$^+$ mice (Fig. 1). HPLC analyses

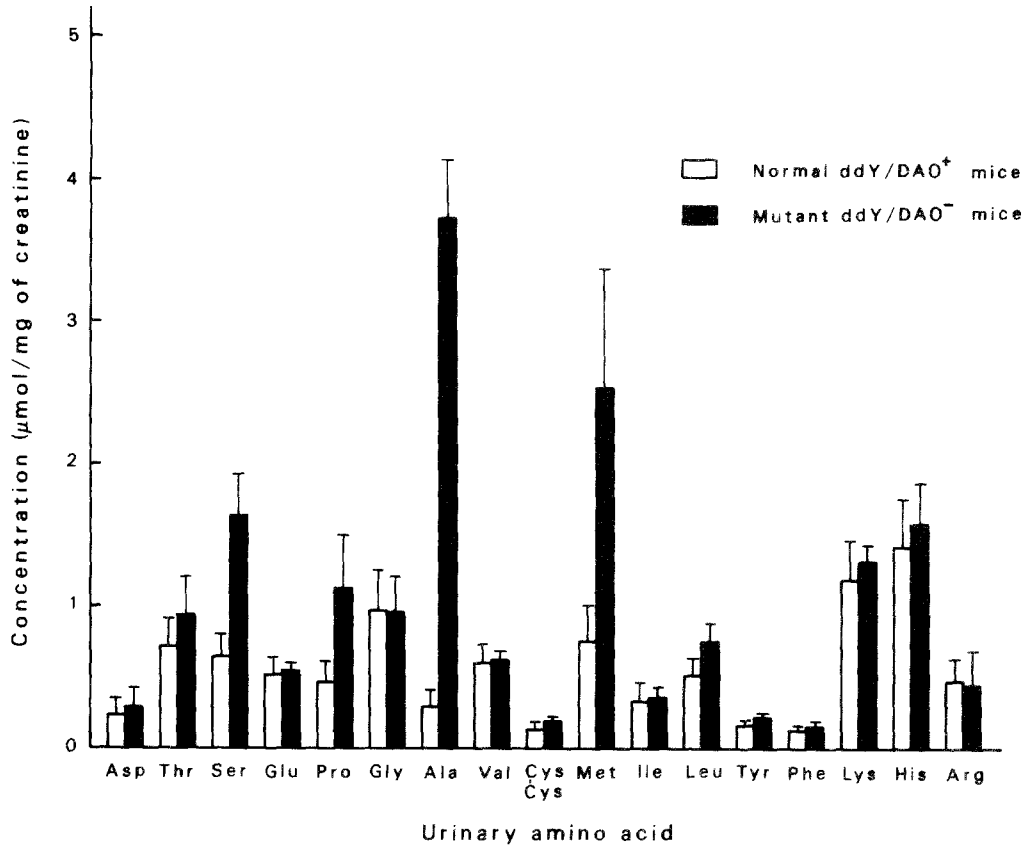


Fig. 1. Amino acid concentrations in mouse urine. Amino acids were purified from urine by deproteinization and cation-exchange chromatography. Their concentrations were determined using an amino acid analyzer. Open column: normal ddY/DAO$^+$ mice. Closed column: mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity. Bars show S. D.

showed that most of these amino acids were D-isomers. The D-alanine was considered to come from cell walls of intestinal bacteria (Konno et al., 1989, 1990) and the D-methionine was determined to originate from DL-methionine supplemented in the commercial mouse diet (Konno et al., 1988b).

In addition to the alanine and methionine, urine of the ddY/DAO$^-$ mice contained more serine (about 2.6 times) and proline (about 2.5 times) than that of the ddY/DAO$^+$ mice (Fig. 1). To examine the nature of the serine and proline, amino acids were purified from urine of the ddY/DAO$^-$ mice, treated with hog kidney D-amino-acid oxidase and subjected to amino acid analysis. Figure 2 shows that the treatment with the oxidase decreased alanine and methionine to the normal levels. This result is consistent with our previous results that most of these amino acids were D-isomers. Figure 2 also shows that the serine and proline were reduced by the treatment to the normal levels as well. This result suggests that the extra portions of the urinary serine and proline consist of D-isomers.

Serine was purified from urine of the ddY/DAO$^-$ mice by cation- and anion-exchange chromatography, followed by thin-layer chromatography. The

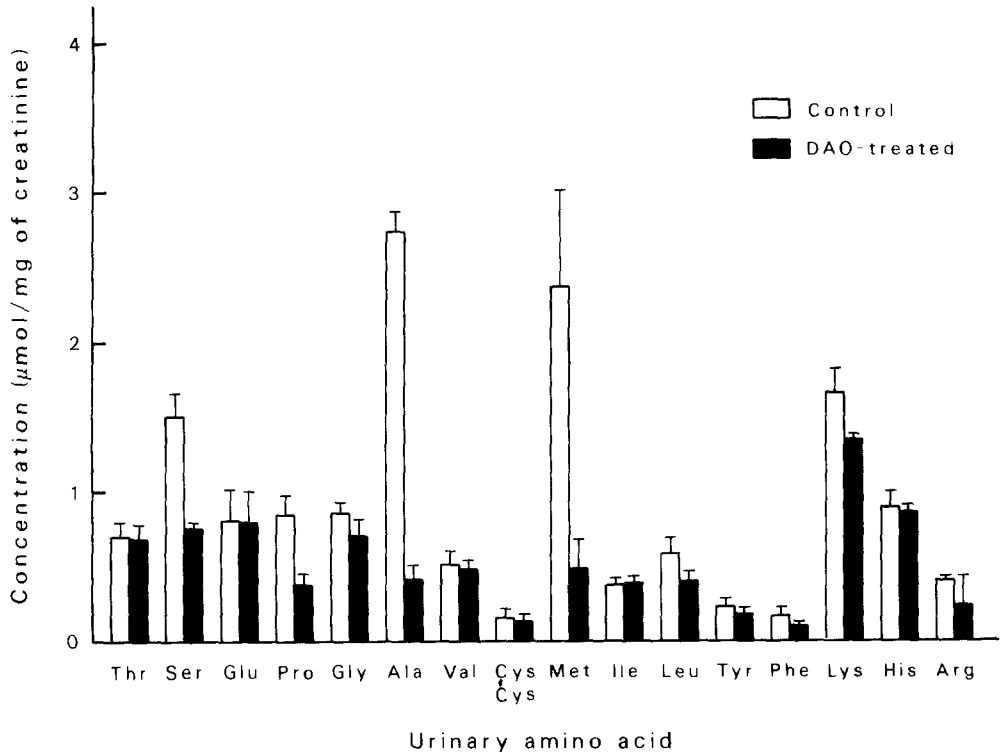


Fig. 2. Change of urinary amino acid concentrations after D-amino-acid oxidase treatment. Amino acids were purified from urine of mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity by deproteinization and cation-exchange chromatography. They were incubated with or without D-amino-acid oxidase. After deproteinization and cation-exchange chromatography, amino acid concentrations were determined. Open column: urinary amino acids without D-amino-acid oxidase treatment (control). Closed column: urinary amino acids treated with D-amino-acid oxidase. Bars show S. D.

exact isomer composition of the serine was determined using an HPLC equipped with a chiral column. A representative chromatogram is shown in Fig. 3. Consistently with the above result, most of the serine had the D-configuration.

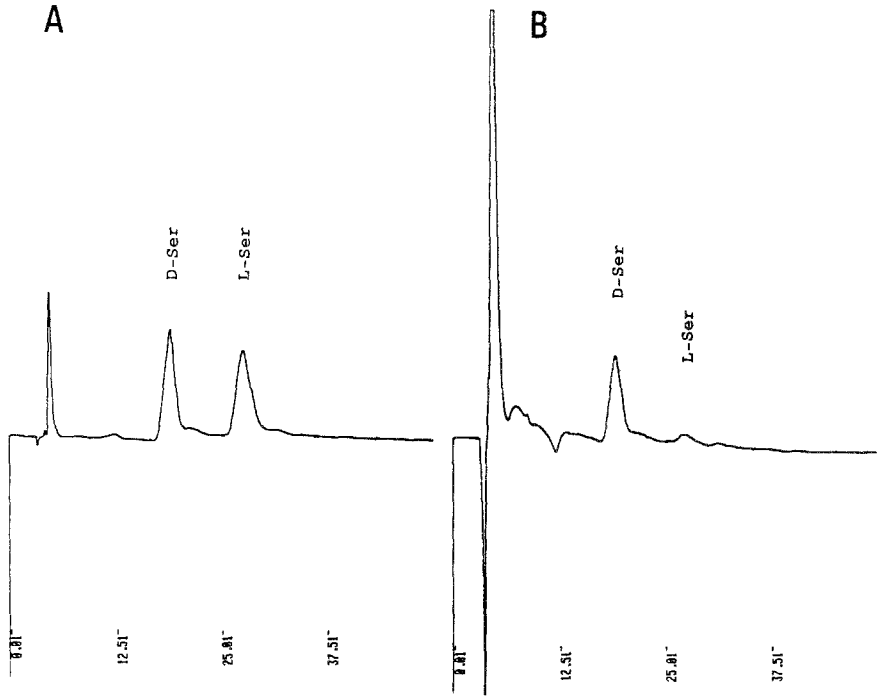


Fig. 3. Analysis of D- and L-composition of urinary serine. Serine was purified from urine of ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity by deproteinization, cation- and anion-exchange chromatography, followed by thin-layer chromatography. D- and L-Serine was separated by high performance liquid chromatography. **A** standard DL-serine. **B** serine purified from urine of the ddY/DAO$^-$ mice

The ddY/DAO$^-$ mice were raised on the Type NMF diet. This diet contained DL-methionine as a supplement. The relationship between the D-methionine and the urinary D-serine and D-proline was then examined. When the ddY/DAO$^-$ mice were fed Rodent Laboratory Chow 5001 which contained less supplementary DL-methionine or the modified NMF diet which did not contain supplementary D-methionine, urinary methionine was reduced to the normal level as observed before (Fig. 4; 1988b). However, contents of serine and proline as well as alanine were not reduced (Fig. 4). These results indicate that the urinary D-serine and D-proline were not related to the D-methionine supplemented in the diet. Actually, there is no evidence for the presence of metabolic pathways which convert D-methionine to D-serine or D-proline.

The ddY/DAO$^-$ mice were given water containing antibiotics (amoxicillin and minocycline) for a week to suppress intestinal bacteria. Then, urine was collected and measured for amino acids. Figure 5 shows that urinary alanine was decreased to the normal level as observed before (Konno et al., 1990). However, the contents of serine and proline as well as methionine were not

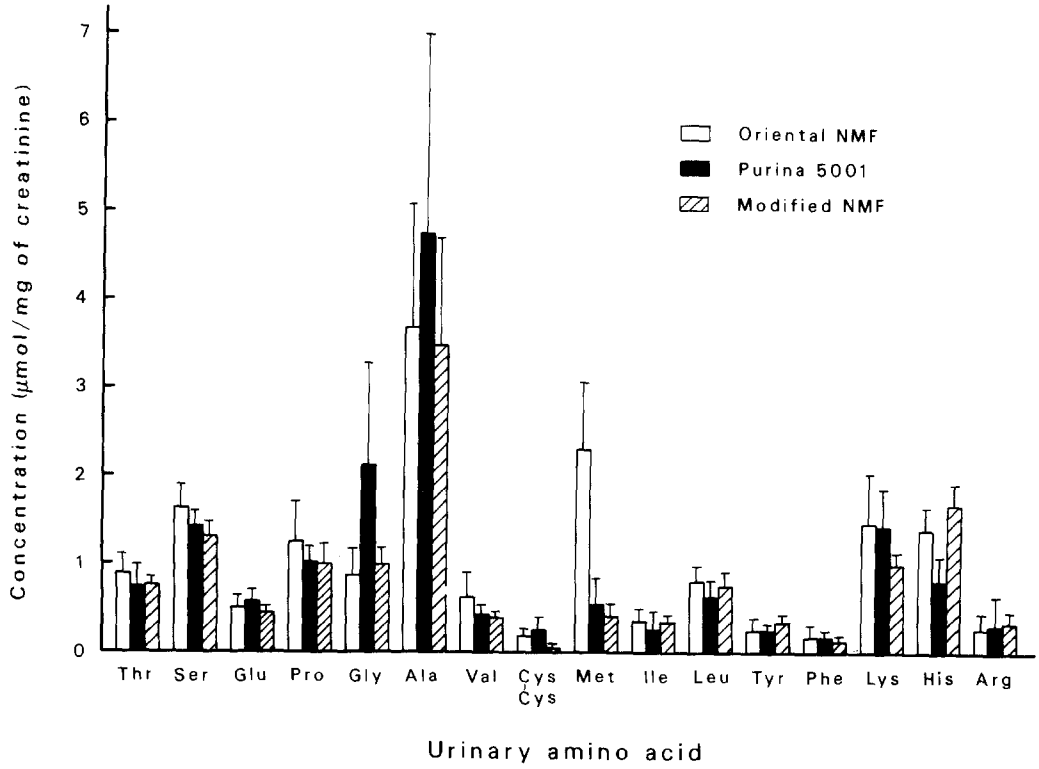


Fig. 4. Effects of diets on urinary amino acid concentrations in ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity. The mice were fed three different diets and their urine were examined for amino acid concentrations. Open column: Type NMF diet from Oriental Yeast Co., which contained supplementary DL-methionine. Closed column: Rodent Laboratory Chow 5001 from Ralson Purina Co., which contained less supplementary DL-methionine. Shaded column: Modified NMF diet which contained no supplementary D-methionine. Bars show S. D.

changed. These results suggest that the D-serine and D-proline in urine of the ddY/DAO$^-$ mice are not of intestinal bacterial origin. The sources of these two D-amino acids are not known at present. Brückner and Hausch (1990) also found that D-serine was present in the plasma of a chronic dialysis patient.

In addition to the amino acids above, urine of the ddY/DAO$^-$ mice contained another ninhydrin-positive substance whose concentration was negligible in urine of the normal mice. Its retention time was very similar to that of methionine sulfoxide and/or methionine sulfone in the amino acid analysis. These two methionine derivatives were not well separated in the amino acid analyzer used. Because their peaks and the peak of threonine partly overlapped, the exact identification and quantitation of the unknown substance have not yet been successful. However, since treatment of urinary amino acids with hog kidney D-amino-acid oxidase decreased its concentration, this substance seems to have D-configuration. When the ddY/DAO$^-$ mice were fed the modified NMF diet which did not contain D-methionine, the substance was not present in their urine. Therefore, it is most likely that the substance is D-methionine sulfoxide and/or D-methionine sulfone derived from D-methionine supple-

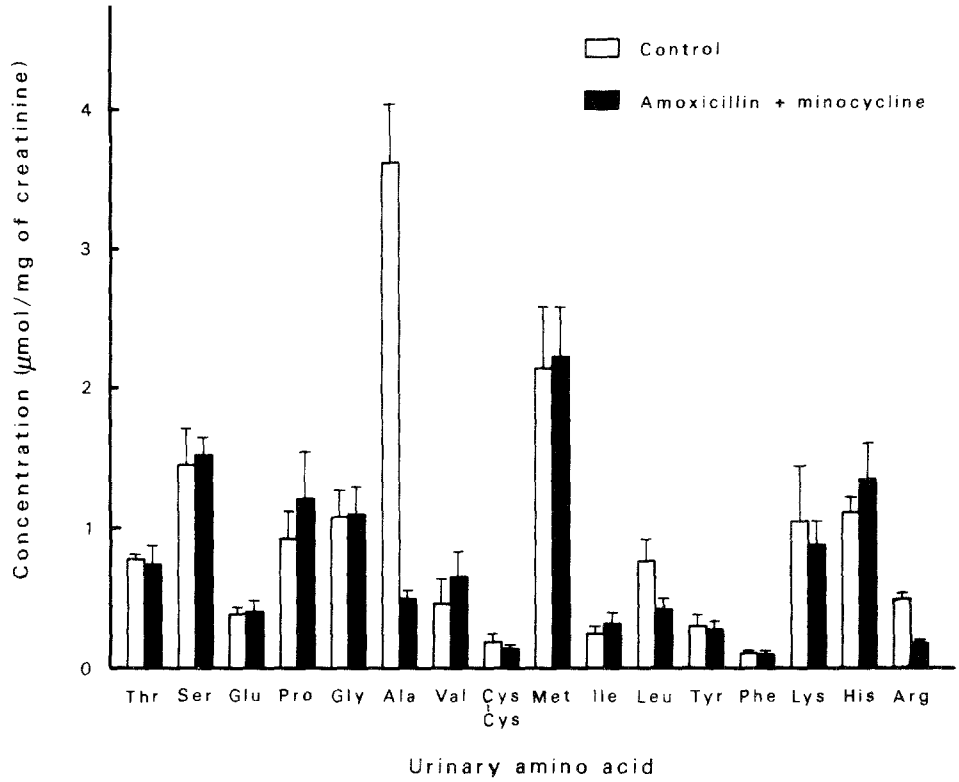
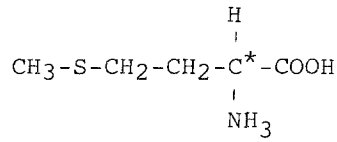
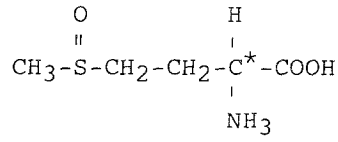


Fig. 5. Effects of oral administration of antibiotics on urinary amino acid concentrations. After ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity were given water containing 0.01% amoxicillin and 0.002% minocycline for a week, their urine was collected and measured for amino acid concentrations. Open column: the mice given plain water (control). Closed column: the mice given water containing the antibiotics

D-Methionine



Methionine sulfoxide



Methionine sulfone

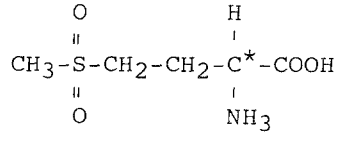


Fig. 6. Structural formula of D-methionine, methionine sulfoxide and methionine sulfone. *C** an asymmetric carbon atom

mented in the diet. It is considered that the ddY/DAO$^-$ mice can not metabolize D-methionine ingested from the diet due to a lack of D-amino-acid oxidase, so they excrete most of it unchanged, and the rest is oxidized to methionine sulfoxide and/or further to methionine sulfone with the retention of the D-configuration (Fig. 6), which is also excreted into urine. It is known that methionine is readily converted to methionine sulfoxide and methionine sulfone (Yang, 1970; Friedman and Gumbman, 1988). The above explanation, however, needs to be verified by identifying the unknown substance.

The mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity manifested a specific renal D-aminoaciduria. This result, on the contrary, indicates that the D-amino acids are constantly metabolized by the oxidase in normal mice. Metabolism of D-amino acids would be one of the physiological roles of D-amino-acid oxidase.

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Authors' address: Dr. R. Konno, Department of Microbiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan.