7 Nagata, Y., Akino, T., Ohno, K., Kataoka, Y., Ueda, T., Sakurai, T., Shiroshita, K., and Yasuda, T., Clin. Sci. *73* (1987) 105.
8 Nagata, Y., Akino, T., and Ohno, K., Analyt. Biochem. *150* (1985) 238.
9 Schleifer, K. H., and Kandler, O., Bact. Rev. *36* (1972) 407.
10 Nagata, Y., Shimojo, T., and Akino, T., Comp. Biochem. Physiol. *91B* (1988) 503.
11 Konno, R., Nagata, Y., Niwa, A., and Yasumura, Y., Biochem. J. *261* (1989) 285.

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D-Aspartate oxidase activity and D-aspartate content in a mutant mouse strain lacking D-amino acid oxidase

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Summary. A mutant mouse strain ddY/DAO$^-$ lacks D-amino acid oxidase activity and accumulates free neutral D-amino acids in its tissues. In this study, D-aspartate oxidase activity and D-aspartate content in the tissues of these mutant mice were compared with those of normal mice. No significant difference was observed, indicating that the metabolism of acidic D-amino acids was unaffected in the mutant.
Key words. D-Amino acid oxidase; D-aspartate oxidase; D-aspartate; mutant mouse.

D-Amino acid oxidase and D-aspartate oxidase in mammals are FAD enzymes with a molecular weight of approximately 39,000. Both are found in peroxisomes in tissues like kidney, liver and brain, and both catalyze the oxidative deamination of D-amino acids. The main difference between the two enzymes is in the substrate specificity; the former oxidizes neutral D-amino acids, whereas the latter oxidizes acidic D-amino acids. This pattern suggests that the two enzymes have complementary roles, although their physiological function remains obscure. In addition to the difference in the substrate specificity, there are clear differences in the inhibitor specificity; benzoate is a potent inhibitor of D-amino acid oxidase[1] without significant effect on D-aspartate oxidase[2,3], whereas meso-tartrate selectively inhibits D-aspartate oxidase[4,5].

Making use of the strict difference in the substrate specificities, D-aspartate oxidase and D-amino acid oxidase activities have been separately assayed in the same sample, with D-aspartate and D-alanine as the substrates[6]. The validity of the method was supported by the finding that the observed activities of the two enzymes were markedly inhibited only by the selective inhibitor of each enzyme[6].

The presence of D-amino acid oxidase in mouse tissues has been known for a long time; it was once reported that the enzyme was absent from the liver[7], but its presence was recently demonstrated by Nagata et al. with sensitive methods to assay activity[8] and an enzyme-linked immunosorbent method[9].

There has been no information on D-aspartate oxidase in mouse tissues before the report by Yamada et al.[10], which showed the presence of the enzyme activity in several tissues including kidney, liver and brain. It was further shown that administration of D-aspartate to mice increased the liver enzyme activity[11]. As expected, the activity was sensitive to meso-tartrate, but not to benzoate.

Recently, Konno et al. established a mutant mouse strain, ddY/DAO$^-$, which lacks D-amino acid oxidase activity[12]. Genetic crosses showed that the mutant carried an autosomal codominant null allele for both kidney and brain enzymes[12]. Moreover, the mutant mice were found to have higher D-amino acid contents and excrete more D-amino acids in the urine than normal mice[13–15]. In view of the close similarity between the two enzymes described above, and the suggestion that there is a complementary relationship, it was quite interesting to us whether D-aspartate oxidase was intact or affected in the mutant strain. In the present study we compared the enzyme activity and D-aspartate content in the tissues of the mutant strain with those in the normal strain.

Materials and methods

The mutant mouse strain ddY/DAO$^-$, lacking D-amino acid oxidase activity, was established by Konno et al.[12] and raised on a stock diet (type NMF; Oriental Yeast, Japan) together with the normal control ddY/DAO$^+$ mice. Male mice (6–7 weeks old) were chosen for the experiment. The mice were killed by decapitation, and the liver, kidney and brain were rinsed with ice-cold saline to remove blood, and stored at -80 °C before use. Assay of D-aspartate oxidase activity was conducted as

Table 1. D-Amino acid oxidase activity in the kidney and D-aspartate oxidase activity in the kidney, brain and liver of mutant ddY/DAO$^-$ and normal ddY/DAO$^+$ mice. Values are mean ± SD for 5 or 6 animals.

Strain	D-Amino acid oxidase activity (nmol/min · mg protein)	D-Aspartate oxidase activity (pmol/min · mg protein)		
	Kidney	Kidney	Brain	Liver
ddY/DAO$^-$ (n = 5)	0.52 ± 0.07	884 ± 132	110 ± 22	47 ± 16
ddY/DAO$^+$ (n = 6)	15.3 ± 1.8	1011 ± 138	126 ± 24	36 ± 6

described elsewhere[10]. For assay of free D-aspartate, the organs were homogenized with 10 vols of 6% perchloric acid at 0 °C in a Potter-Elvehjem homogenizer equipped with a Teflon pestle, and the homogenate was centrifuged at 42,000 × g for 20 min at 4 °C. The supernatant was neutralized with KOH, chilled for 20 min and centrifuged at 4 °C again. The supernatant (2–4 ml) was loaded on a Dowex 1 × 2 (100–200 mesh, acetate form) column (1 ml volume). After washing the column with 10 ml of water, D-aspartate was eluted with 7.5 ml of 1 M acetic acid. The eluent was concentrated to dryness under reduced pressure to remove acetic acid and the residue was dissolved in water containing 40 µM L-cysteic acid.

The sample solution was analyzed by the HPLC method of Aswad[16], with the modification that L-cysteic acid was employed as the internal standard. A Gilson HPLC system was used, and the detector was a Gilson Model 121 fluorescence detector equipped with a 240–410-nm excitation filter and a 430-nm emission filter. Detector response was monitored on a SIC Chromatocorder 12. A reversed phase HPLC column, (4.6 × 100 mm) packed with 3-µm-diameter Cosmosil 3C18, was obtained from Nacalai Tesque Inc. (Kyoto, Japan). The solvent system was 50 mM sodium acetate buffer, pH 5.9 containing 4% (v/v) methanol and the flow rate was 0.8 ml/min. Protein concentration was measured by the method of Lowry et al.[17].

Results

Table 1 shows D-amino acid oxidase activity in the kidney and D-aspartate oxidase activity in the kidney, brain and liver of the mutant ddY/DAO$^-$ and the normal ddY/DAO$^+$ mice. The D-amino acid oxidase activity in the kidney, which is usually the highest in the various mouse tissues, was very low in the mutant mice compared with that in the normal ddY/DAO$^+$ mice. On the other hand, there was no difference between ddY/DAO$^-$ and ddY/DAO$^+$ strains in the D-aspartate oxidase activity of kidney, brain and liver.

Table 2. Free D-aspartate content in the kidney of the mutant ddY/DAO$^-$ and the normal ddY/DAO$^+$ mice. Values are mean ± SD for 5 or 6 animals.

Strain	Free D-aspartate (nmol/g wet wt)
ddY/DAO$^-$ (n = 5)	19.1 ± 3.5
ddY/DAO$^+$ (n = 6)	16.2 ± 9.4

Table 2 shows free D-aspartate content in the kidney of the mutant ddY/DAO$^-$ and the ddY/DAO$^+$ mice. The data indicate that there is no difference between the mutant and the normal mice.

Discussion

The finding that there was no difference between the mutant ddY/DAO$^-$ and the normal ddY/DAO$^+$ mice in the free D-aspartate content of the kidney contrasts with the finding that free neutral D-amino acids accumulated in the mutant mice because of the lack of D-amino acid oxidase[18]. Thus the result confirms that D-amino acid oxidase does not oxidize D-aspartate in vivo, which is consistent with the substrate specificity of this enzyme as found in vitro. The result also indicates that accumulation of neutral D-amino acids does not influence the concentration of D-aspartate in tissues.

As described above, lack of D-amino acid oxidase activity in the mutant mice was not accompanied by any significant decrease of D-aspartate oxidase activity in the kidney, brain and liver. This finding suggests that each enzyme is synthesized independently, although D-amino acid oxidase closely resembles D-aspartate oxidase in function and in amino acid composition[19]. It does not seem possible that D-amino acid oxidase could be a precursor of D-aspartate oxidase. Probably, the genetic loci of the two enzymes are not linked closely. It should also be pointed out that D-aspartate oxidase activity in the mutant did not increase either, despite the accumulation of neutral D-amino acids, although it might have been expected that D-aspartate oxidase might increase to compensate for the lack of D-amino acid oxidase. This result is compatible with the report that D-aspartate oxidase activity was increased by D-aspartate administration, but not by D-alanine administration[11].

In conclusion, the results seem to indicate that the metabolism of D-aspartate and the activity of D-aspartate oxidase are independent of the metabolism of neutral D-amino acids and the activity of D-amino acid oxidase. D-aspartate oxidase may have a function distinct from that of D-amino acid oxidase, as suggested by Hamilton[20].

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1 Klein, J. R., and Kamin, H., J. biol. Chem. *138* (1941) 507.
2 Still, J. L., Buell, M. V., Knox, W. E., and Green, D. E., J. biol. Chem. *179* (1949) 831.

3 Still, J. L., and Sperling, E., J. biol. Chem. *182* (1950) 585.
4 De Marco, C., and Crifo, C., Enzymologia *33* (1967) 325.
5 Dixon, M., and Kenworthy, P., Biochim. biophys. Acta *146* (1967) 54.
6 Yusko, S. C., and Neims, A. H., J. Neurochem. *21* (1973) 1037.
7 Shack, J., J. natl Cancer Inst. *3* (1943) 389.
8 Nagata, Y., Shimojo, T., and Akino, T., Comp. Biochem. Physiol. *91B* (1988) 503.
9 Nagata, Y., and Akino, T., Comp. Biochem. Physiol. *89B* (1988) 179.
10 Yamada, R., Nagasaki, H., Wakabayashi, Y., and Iwashima, A., Biochim. biophys. Acta *965* (1988) 202.
11 Yamada, R., Nagasaki, H., Wakabayashi, Y., and Iwashima, A., Biochim. biophys. Acta *990* (1989) 325.
12 Konno, R., and Yasumura, Y., Genetics *103* (1983) 227.
13 Konno, R., Isobe, K., Niwa, A., and Yasumura, Y., Biochim. biophys. Acta *967* (1988) 382.
14 Konno, R., and Yasumura, Y., Lab. Anim. Sci. *38* (1988) 292.
15 Konno, Y., Nagata, Y., Niwa, A., and Yasumura, Y., Biochem. J. *261* (1989) 285.
16 Aswad, D. W., Analyt. Biochem. *137* (1984) 405.
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
18 Nagata, Y., Konno, R., Yasumura, Y., and Akino, T., Biochem. J. *257* (1989) 291.
19 Negri, A., Massey, V., and Williams, Jr, J. biol. Chem. *262* (1987) 10026.
20 Hamilton, G. A., in: Advances in Enzymology, vol. 57, p. 85. Ed. A. Meister. Academic Press, New York and London 1985.

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Creatol (5-hydroxycreatinine), a new toxin candidate in uremic patients

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Summary. Both 5-hydroxy-1-methylhydantoin (**3**) and the hitherto unrecognized 5-hydroxycreatinine (2-amino-5-hydroxy-1-methyl-imidazol-4(5*H*)-one or creatol) (**6**) can be isolated from the urine of uremic patients, in whom these compounds probably arise as oxidative metabolites of creatinine (**1**). The enhanced production of the well-known uremic toxin, methylguanidine (**8**), from creatinine (**1**) in such patients, almost certainly occurs via the newly recognized metabolite (**6**).
Key words. Creatinine; 5-hydroxycreatinine; creatol; 5-hydroxy-1-methylhydantoin; uremic toxin; uremia.

During our investigation of novel metabolites produced in animals under abnormal conditions such as viral infection, we isolated two hydantoins, 1-methylhydantoin (**2**) and its 5-hydroxy derivative (**3**), which eventually proved to be bio-active as plant growth regulators[1]. Although neither hydantoin has been detected in normal animals, we recently showed that mammals do have the capacity to metabolize (**2**) into (**3**) and thence into methylurea (**5**)[2,3], as represented in figure 1. We therefore concluded that the production of the new metabolite (**3**) in infected animals probably depended on the formation[4,5] of its precursor (**2**) from creatinine (**1**) under the abnormal conditions pertaining. Hence, we expected to find evidence for the metabolite (**3**) in uremic patients, who were already known[4] to be capable of converting creatinine (**1**) into the precursor (**2**).

A greatly increased production of the uremic toxin[6,7] methylguanidine (**8**) has been observed in mammals with chronic renal failure, and creatinine (**1**) has been implicated as one of its precursors. The involvement of 'active oxygen' in the oxidative conversion of creatinine (**1**) into methylguanidine (**8**) has been demonstrated[8,9], as has the increased capacity of uremic mammals to produce such active species[10]. However, the mechanism by which the production of (**8**) is increased[6,11,12] in uremic mammals has remained an open question. Seeking to answer this question, we now postulate an alternative oxidative pathway, operative in uremic mammals, for the conversion of creatinine (**1**) to methylguanidine (**8**) via 5-hydroxycreatinine ('creatol': **6**) and perhaps 2-amino-1-methyl-1*H*-imidazole-4,5-dione (**9**) (fig. 1).

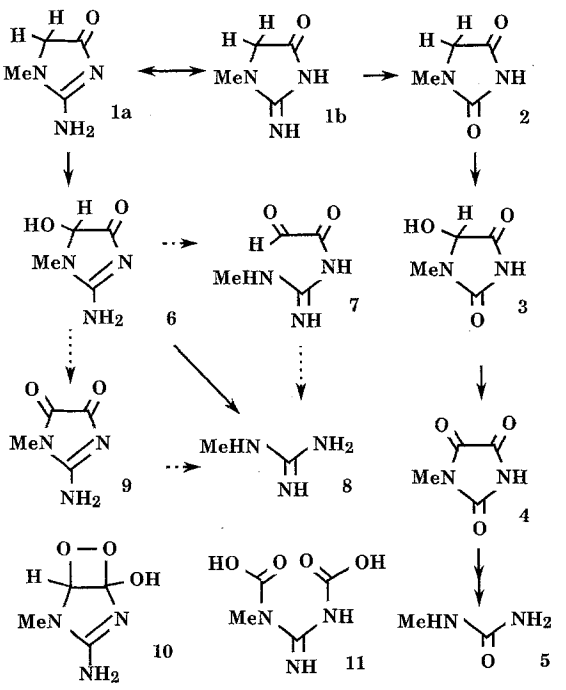

Figure 1. The metabolic pathway of creatinine (**1**) in uremia.