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D-Amino acids in mouse tissues are not of microbial origin

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Summary. Neutral free D-amino acid contents in the serum, kidney, liver, brain, small intestine and urine in germ-free mice and those in specific pathogen-free mice were compared. No significant difference was found. This strongly suggests that the free D-amino acids which were shown to be present in mice in our previous work [1, 2] did not originate from the enteric microbial flora.
Key words. D-Amino acid; microflora; germ-free; mice.

It has long been believed that the naturally occurring amino acids have an L-configuration in mammals [3]. Only D-aspartic acid is known to accumulate in metabolically stable proteins in humans, as a result of in vivo racemization [4–6]. However, we have observed considerable amounts of free D-amino acids (DAA) in plasma from patients with renal diseases [7], and in the kidney [2] of a mutant mouse lacking D-amino acid oxidase, using a sensitive enzymic method for estimating neutral DAA [1, 8]. Free DAA were also detected in normal human plasma [7], and in normal mouse kidney, liver, brain, heart, lung, thymus and serum [1]. Recently, the presence of free DAA has been confirmed by high performance liquid chromatography in the serum and in kidney extracts from a mutant mouse. D-isomers of alanine, proline, serine and glutamine were detected (Nagata et al., unpubl. obs.).

Hoeprich [3] resolved the paradox that *Mycobacterium tuberculosis* was unsusceptible to D-cycloserine in the treatment of experimental tuberculosis in guinea pigs and mice, although it was susceptible to the drug on testing in vitro, by detecting D-alanine, an antagonist to the antibiotic action of D-cycloserine, in the serum of these animals. He thus provided the first documentation of the occurrence of a free D-amino acid in the blood of mammalian species. However, his conclusion was that the D-alanine in guinea pigs and mice was probably from an exogenous source, most likely from the enteric microflora, for no D-alanine had been detected in the serum of guinea pigs and mice from germ-free colonies. His view is still the prevailing one.

In order to investigate the source of DAA, we measured free DAA levels in the serum, kidney, liver, brain, small intestine and urine from bacteria-free mice, and compared them with those from normal control mice. The result was in contrast to that of Hoeprich, i.e., no difference was observed between the two kinds of mice. Therefore, the source for DAA is not likely to be the microbial flora of the intestine. An interpretation of the discrepancy is given under 'Results and discussion'.

Materials and methods

Male germ-free (GF) mice (ICR, 6 weeks old) and age- and sex-matched specific pathogen-free (SPF) mice of the same strain were obtained from Japan Clea (Tokyo). They were maintained in the Animal Care Center of Sapporo Medical College for 15 days as follows. Each animal was housed alone in an autoclave-sterilized plastic cage with a mesh floor, in order to prevent the mice from having access to feces, because mice may be coprophagous, and the feces of an SPF mouse contain many bacteria. D-alanine and D-glutamic acid are known to be components of the bacterial cell wall [9]. The cages were kept in a vinyl isolator, which was freshly sterilized. The mice were fed autoclaved NIH-improved type chow (Oriental Yeast, Tokyo) which included no supplemental DAA, and supplied with autoclaved water. The GF mice were confirmed to be axenic until the last day of breeding, by culture tests of specimens collected from the diet chow, drinking water and feces, and the inner surfaces of the cages, on days 0, 7, 11 and 15. The specimens were cultured in thioglycollate medium for 7 days at 37 °C as well as at room temperature, and in glucose-peptone medium for 10 days at room temperature. The SPF mice were shown by the culture tests to have been infected with both aerobic and anaerobic bacteria, and eumycetes.

Blood was collected from axillary vessels under anesthesia with ether, after 16–19 h starvation, and the serum was separated by centrifugation after clotting at room temperature. The kidney, liver, small intestine and brain

were isolated and rinsed with phosphate-buffered saline, pH 7.4. Each organ was minced with scissors, and homogenized with 4 vols of phosphate-buffered saline in a glass homogenizer in an ice bucket, at 1100 rpm for 1 min. The homogenate was centrifuged at 160,000 × g for 10 min at 4 °C. The supernatant fluid, the extract, was taken for DAA analysis. When not in use, the extracts and serum were frozen at − 20 °C.

Measurement of free neutral DAA was carried out with the enzymic method previously reported [1, 8]. Urinary creatinine was determined with a kit (Serotec, Sapporo).

Results and discussion

The table clearly shows that there is no difference in the free DAA level between the GF and SPF mice. Similar free DAA levels to the present ones have also been observed in BALB/c mice raised under SPF-condition [1]. According to Hoeprich [3], however, the D-alanine content of mouse (Swiss-Webster albino) serum was 150 (range 50–420) nmol/ml; the value is 5-fold higher than the present result (table). Such a conspicuously high level of DAA has only been detected in a bacteria-contaminated sample in our experience. Hence, it is suspected that the sample assayed by Hoeprich might have been considerably contaminated with microbes. In our experiments care was taken to sample solutions for detection of possible microbial contamination. On the other hand, no DAA was detected by Hoeprich [3] in the serum from GF mice (range 0–10 nmol/ml). In his report only values greater than 50 nmol/ml were considered to indicate significant quantities of D-alanine. In the present study, an accurate measurement of 0.5 nmol/ml was possible with the enzymic method [1, 8]. If the sera from the GF mice examined by Hoeprich had been analyzed by the present method, an appreciable amount of DAA would probably have been measured.

Thus it has been demonstrated that the free neutral DAA, detected in mouse tissues in our previous [1, 2] and present experiments, did not originate from microbes. The food is unlikely to be a significant candidate for the source of free DAA because, firstly, a special diet not including any supplemental DAA was fed to the mice in the present experiment (the daily amount of DAA taken through the diet chow was about 5.6 μmol); secondly, care was taken to prevent the mice from having access to their feces by using the mesh floor; and lastly, in an experiment where mice took orally as much as 1.95 mmol of D-alanine a day for 15 days, the serum-free DAA level elevated only to 81 nmol/ml (Nagata et al., unpublished results).

Therefore, the view appears possible that the free DAA are from amino acid residues of proteins which constitute or function in the mouse body. In the case of metabolically stable proteins such as human tooth enamel [4] and dentine [5], and lens protein [6], D-aspartic acid residues that have arisen by chemical racemization are known to accumulate in the protein with age. The same thing may be taking place in neutral amino acid residues in metabolically active proteins. The resultant DAA might be liberated from the proteins in various tissues into blood, and the free DAA produced may be decomposed by D-amino acid oxidase situated in the kidney, liver and other tissues [10]. This postulate is strongly supported by the fact that the free DAA levels of various tissues, including serum, were 2- to 6-fold higher in the mutant mice than the control mice [2]. Free DAA are also eliminated from the body by excretion [2, 11] into the urine. As a consequence, small quantities of free DAA exist in normal mice. The highest free DAA level of the serum from mutant mice lacking D-amino acid oxidase, around 50 nmol/ml [2], may indicate the DAA level, that develops in the blood under conditions where there is no effect of D-amino acid oxidase.

The sera were analyzed with high performance liquid chromatography in attempts to confirm the present experimental results and to obtain information on individual amino acids of the D-configuration. However, the ratio of DAA to total free amino acids was not high enough to give accurate results in the normal mice, owing to the activity of D-amino acid oxidase, and hence the data are not shown.

To conclude, a low level of free neutral DAA in mouse serum, less than 50 nmol/ml, is thought to have originated from the mouse's own proteins, whereas DAA present in amounts greater than 50 nmol/ml may be regarded as exogenous ones from bacterial cell wall or other microbes. The DAA in the mouse kidney, liver, brain, small intestine and urine could also possibly be considered endogenous.

Free neutral DAA levels in serum, kidney, liver, brain, small intestine and urine of GF and SPF mice. Values are mean ± SEM of four observations. In each of the four observations a different animal was used. g, wet weight of the tissues; Cr, creatinine.

	Free D-amino acids	
	SPF mice	GF mice
Serum (nmol/ml)	33.4 ± 3.5	27.3 ± 4.1
Kidney (nmol/g)	94.8 ± 19.2	101.8 ± 16.4
Liver (nmol/g)	146.5 ± 34.0	150.8 ± 43.5
Brain (nmol/g)	111.3 ± 16.1	90.2 ± 19.3
Small intestine (nmol/g)	115.4 ± 32.3	179.4 ± 40.3
Urine (nmol/mg Cr)	1494 ± 174	1313 ± 319

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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