Urinary Excretion of D-Serine in Human: Comparison of Different Ages and Species

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The urinary excretion of D-serine (D-Ser) in human, rat and dog of various ages was studied. Great amounts of D-Ser were consistently excreted in human urine throughout life. No age-dependent changes were observed in urinary D-Ser/total-Ser ratios from the newborn to the aged. D-Ser/creatinine ratios in adult human urine were found to be relatively constant in individuals. The constant excretion of D-Ser in human urine was confirmed by analyzing the consecutive 24 h urine of three volunteers. High concentrations of D-Ser and D-alanine (D-Ala) were found in adult dog urine. The urinary D-Ser concentration was high in young rats at unweaned and weaned periods, and then declined with increasing age. In contrast, the urinary D-Ala concentration was very low in suckling rats, and increased rapidly after the weaned state and then declined with increasing age. The species- and age-related excretion of D-Ser in mammalian urine is considered to be due to the differences in the renal handling of D-Ser, because plasma D-Ser concentrations among the groups were not so different. Although free D-Ser has been detected in animal foods and human colostrum, the amount is insufficient to explain the concentration of D-Ser found in urine. These results indicate that urinary D-Ser in mammals may be mainly of endogenous origin.

Key words D-serine; D-alanine; urinary excretion; D-amino acid oxidase; food; kidney

It has long been believed that proteins and free amino acid pools in mammals consist of only L-amino acids. With the development of enantiomeric separation technology, however, D-amino acids have recently been found in tissues and biological fluids of mammals.²⁾ Among free D-amino acids found in the mammalian body, D-serine (D-Ser) seems to be physiologically important because a high concentration of free D-Ser is consistently present in the forebrains of human and other mammals throughout life,^{3–5)} and its distribution in rat brain is closely correlated with N-methyl-D-aspartate (NMDA) receptors.^{3,6,7)} Furthermore, D-Ser is known to be a potent agonist for NMDA receptors *in vitro* and *in vivo*.^{8,9)} Therefore, it is now proposed that D-Ser is an intrinsic modulator of NMDA receptors in the brain.^{3,6,7)} On the other hand, there are no significant changes in the free D-Ser concentrations in the brain of patients with Alzheimer's, Parkinson's and schizophrenia.^{10–12)} Unchanged brain D-Ser concentration in ataxic mutant mice has also recently been reported.¹³⁾ The metabolic fate of D-Ser remains unknown, though some studies have suggested that D-amino acid oxidase may be involved.^{14,15)}

Free D-Ser concentrations in peripheral tissues were much lower than those in the brain.¹⁶⁾ However, Brückner *et al.* recently found, using GC-MS and HPLC methods, that free D-Ser was present in urine from adult humans at a high concentration.¹⁷⁾ This finding seems to be noteworthy since D-Ser is a well-known as nephrotoxic agent that causes acute necrosis of the proximal tubules when administered to rats at a high dose.^{18–21)} The mechanism of the nephrotoxicity is not understood. The origins of D-Ser in urine as well as in the brain are not known either. We wonder whether there is any relationship between the urinary D-Ser and brain D-Ser. In an attempt to explore the biological significance and origin of urinary D-Ser in mammals, we investigated the urinary D-Ser ex-

cretion in human, rat and dog of various ages by utilizing fluorometric HPLC methods developed in this laboratory.^{22–24)} The free D-Ser content in animal foods and human colostrum was also determined in order to distinguish whether urinary D-Ser is of dietary origin. For comparison, urinary D-alanine (D-Ala) levels were also determined.

MATERIALS AND METHODS

Material 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and Creatinine Test Wako were obtained from Wako Pure Chem., Japan. Standard D-/or L-amino acids were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All other reagents and solvents were of analytical reagent grade.

Animals and Human Samples Specific pathogen-free male Wistar rats with different ages, from Charles River Japan, Inc., were used. Adult male Beagle dogs (from Nihon Nousan Co., Hamamatsu), weighing 14 and 10 kg, were also used. Animals were allowed access to standard dry food and water *ad libitum*. Two-week-old (14 d) rats were nursing. From the 21st day, the rats were separated from their mother and provided an adult diet. Rat blood was drawn from the jugular vein and dog blood was drawn from the brachial vein with heparin as an anticoagulant. Plasma was isolated by centrifugation at 3000 rpm for 5 min. Urine samples from rat and dog were collected for 24 h in metabolism cages while the animals freely ingested water and food. For sterilization, about 100 μ l of toluene was added to the urine collecting container. The urine samples collected were passed through filter papers and stored at -20°C until analysis. In the case of the 2-week-old rats, voiding was either spontaneous or encouraged by gentle suprapubic pressure. Adult human urine was collected from healthy volunteers of different ages at various times. Urine samples of infants and children without

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renal and liver disease were obtained from The Branch Hospital, The University of Tokyo. Healthy adult human serum samples were supplied by The University of Tokyo Hospital. Human colostrum samples from postpartum women were obtained from Nihon University Hospital.

Oral Administration of D-Ser to Rats D-Ser (10 mg/ml in saline) was administered by an intragastric tube into 9-week-old male Wistar rats at a dose of 10 mg/kg. Twenty-four hour urine was then collected from the rats; they were not given any food during that time but were allowed to take water freely. D-Ser recovery from the 24 h urine was calculated as follows: D-Ser amounts in 24 h urine after dosing minus control/D-Ser amounts dosed.

Analysis of Free D-Amino Acids in Animal Foods and Human Colostrum The dried rat food (EQ from PMI Feeds, Inc., St. Louis, U.S.A.) and dried dog food (ED-1 from Sanwa Chemical Co., Japan) used for the feeding of the animals were analyzed for free D-amino acids. Each food (2 g) was moistened with 20 ml distilled water and ground into a small powder. One ml of the food suspension was transferred into a 50 ml centrifuge tube containing 9 ml of methanol, then mixed vigorously. After centrifugation at 10000 rpm for 5 min, the supernatant was derivatized with NBD-F, and free D-Ser and D-Ala were analyzed as described below. Free D-Ser and D-Ala contents in human colostrum were determined by the method described below.

Sample Pretreatment Plasma, milk or urine (20 μ l) was mixed vigorously with methanol (180 μ l), and free amino acids were extracted by centrifuging the mixture at 10000 rpm for 5 min. The supernatant (20 μ l) was transferred to a brown microtube to which was added 20 μ l of 50 mM borate buffer containing 4 mM EDTA (pH 8.0) and 30 μ l of 50 mM NBD-F (in acetonitrile); the mixture was then heated at 60 °C for 2 min. To the reaction mixture was added 70 μ l of 1.0% acetic acid in methanol, then the new mixture was filtrated through a 0.5 μ m membrane filter (Column Guard LCR 4, Nihon Millipore, Tokyo, Japan). The sample (20 μ l) was injected into HPLC as described below.

HPLC Conditions Two Hitachi HPLC systems were used. One HPLC set consisted of an L-6200 Intelligent Pump, a 7161 injector (Rheodyne, Cotai, CA, U.S.A.), F-1000 Fluorometric Detector and D-2500 Chromato Integrator, was used for the isolation of individual amino acids. Another HPLC set consisted of an L-7100 Intelligent Pump, a L-7480 FL-Detector and a D-7500 Chromato Integrator was used for the chiral separation of the isolated amino acids. NBD-Ser and NBD-Ala were determined and isolated by reversed phase HPLC developed by us with a slight modification.²²⁻²⁴ The column was TSK gel ODS 80Ts (250 mm $\times$ 4.6 mm i.d.) obtained from Tosoh, Tokyo. The mobile phase consisted of eluent A, 10% CH₃CN/H₂O (v/v) containing 0.1% trifluoroacetic acid (TFA) and eluent B, 90% CH₃CN/H₂O (v/v) containing 0.1% TFA, which was eluted with a linear gradient programmed at 0–35 min, B, 10%; 35–60 min, B, 10–100% and 60–70 min, B, 100%. The flow rate was kept at 0.8 ml/min. Fluorometric detection was at 530 nm with excitation at 470 nm. The isolated amino acid fractions which contained the D and L isomers were then subjected to a Pirkle-type chiral column for determining the D/L ratio. The chiral column was Sumichiral OA-2500 (R) (Supplied by Sumika Analytical Center, Osaka, Japan). An iso-

cratic elution was performed with 25 mM ammonium acetate in methanol at a flow rate of 1.0 ml/min. Fluorometric detection was carried out as described above. To confirm the D- and L-amino acids, each isolated amino acid fraction was also analyzed by Sumichiral OA-2500 (S) and Sumichiral OA-4700 (S, R) columns with a mobile phase of 7.5 mM citric acid in methanol.²³ The other HPLC conditions were the same as above.

Urine Creatinine (Cr) Test Urinary Cr was determined by a Jaffé reaction according to the instructions of the Creatinine Test Wako.

Statistics The significance of difference between any two groups was assessed by Student's *t*-test.

RESULTS

D-Ser Concentrations in Human Urine Free amino acids in urine were extracted by methanol and then derivatized with NBD-F. The NBD-derivatized amino acids were determined fluorometrically and isolated by reversed phase HPLC as shown in Fig. 1. Each fraction of NBD-Ser and NBD-Ala was collected and subjected to the Sumichiral OA-2500 (R) column for determining its D/L value.^{23,24} Figure 2 shows the chromatograms of D-Ser and L-Ser in human urine. The D-Ser and L-Ser peaks were confirmed by the Sumichiral OA-2500 (S) and the Sumichiral OA-4700 (S, R) columns.²³ The D/L ratios determined in those columns were the same (data not shown). The identification of D-Ala and L-Ala peaks and its D/L ratios were also performed by the Sumichiral OA-series columns. Table 1 summarizes D-Ser and D-Ala concentrations in human urine. Since amino acid concentrations in untimed urine can be affected by urine dilution, the relative concentration compared with urinary Cr was calculated. Though the urinary concentration of D-Ser and the D/D+L ratio were variable in individuals, the D-Ser/Cr value was

Fig. 1. Isolation of NBD-Ser and NBD-Ala in Human Urine by Reversed Phase HPLC

HPLC conditions were as described in the Materials and Methods section.

Fig. 2. Chiral Separation of D-Ser and L-Ser in Human Urine

A: standard racemic-Ser; B: human urinary Ser isolated in Fig.1. HPLC conditions: Sumichiral OA-2500(R) column; mobile phase, 25 mM ammonium acetate/methanol. Other conditions were as described in the Materials and Methods section.

found to be relatively constant in adult human urine.

In an attempt to investigate the within-day variation of D-Ser excretion in human urine, we measured the D-Ser concentration in consecutive 24 h urine from three adult volunteers. As shown in Table 2, urinary D-Ser concentrations varied greatly within-day, and the D-Ser/total-Ser ratio also exhibited some diurnal variation, but the D-Ser/Cr value was relatively constant. The regression coefficients between D-Ser and Cr concentrations in the urine from the three volunteers were 0.98, 0.95 and 0.92, respectively. The diurnal variation of D-Ser/Cr values was even less than that of the L-Ser/Cr values. These results suggest that the urinary excretion of D-Ser in human was little affected by dietary intake. In contrast, the individual and diurnal variations of D-Ala/Cr values were greater than those of the D-Ser/Cr values (Tables 1 and 2).

With regard to age, a great amount of D-Ser was consistently excreted in human urine throughout life (Table 1). No age-dependent changes were observed in urinary D-Ser/total-Ser ratios from newborn infants to the elderly. Although urinary D-Ser/Cr ratios were rather higher in infants than in children and adults, it should be pointed out that this may be partially due to the lower Cr excretion in infants. It is of interest that a considerable amount of D-Ser was excreted into 4 d-old infant urine, who was, due to vomiting, not taking any food but was only being intravenously infused with a solution of glucose and electrolytes after birth.

Table 1. Urinary D-Ser and D-Ala Concentrations in Human at Different Ages

Subject No.	Age (Y)	Sex	D-Ser (μ M)	D-Ser/total-Ser (%)	D-Ala (μ M)	D-Ala/total-Ala (%)	Cr (mg/dl)	D-Ser/Cr (nmol/mg)	D-Ala/Cr (nmol/mg)
1	3 d	M	76	23.3	3	1.5	22	345	14
2 ^{a)}	4 d	M	73	49.1	3	4.7	17	429	18
2 ^{a)}	10 d	M	284	44.6	97	23.2	34	835	285
3	4 months	M	62	46.9	2	2.6	14	450	14
4	6 months	F	68	61.6	11	15.8	22	309	50
5	10 months	M	77	48.1	35	21.3	32	241	109
6	11 months	M	56	48.4	4	5.3	16	350	25
7	1	M	79	24.9	21	5.5	32	247	66
8	1	M	62	45.5	28	23.1	35	177	80
9	3	F	122	34.4	88	39.3	47	260	187
10	4	M	229	50.5	20	12.0	114	201	18
11	5	F	109	45.0	21	17.5	85	130	24
12	8	F	76	42.4	14	10.1	89	85	16
13	9	F	107	43.5	21	13.6	68	157	31
14	13	F	90	24.1	10	6.3	92	98	11
15	14	M	113	47.6	37	20.7	82	138	46
16	15	F	162	54.6	19	13.7	140	116	14
17	18	M	127	45.4	23	12.5	148	86	16
18	22	M	171	18.3	73	9.8	171	100	43
19	24	M	80	20.5	19	7.7	98	82	19
20	25	M	116	51.5	39	16.4	130	89	30
21	29	M	166	34.9	25	6.5	191	87	13
22	31	M	225	31.5	91	18.0	140	161	65
23	32	M	113	44.7	42	20.9	113	100	37
24	32	F	174	30.8	51	14.8	136	128	38
25	33	M	246	39.0	83	12.7	223	110	37
26	42	M	95	53.6	62	21.6	123	77	50
27	55	M	104	34.9	75	21.0	121	86	62
28	70	M	125	40.4	186	45.5	98	127	189
29	75	M	157	28.0	87	15.9	119	132	73
30	76	M	95	48.9	29	14.8	84	119	35
31	78	F	39	74.1	8	13.1	46	85	17
32	94	M	155	52.8	116	21.8	130	119	89

a) These two values correspond to the same person. Due to vomiting, the newborn did not take any food but received intravenous infusions of glucose and electrolyte solution until the 4th day after birth. One urine sample was collected at the 4th day. Thereafter, he began taking maternal milk. Another urine sample was collected at the 10th day.

Table 2. Diurnal Variations of Urinary Excretion of D-Ser and D-Ala in Three Healthy Volunteers

Subject No.	Urine collecting time	D-Ser (μM)	L-Ser (μM)	D-Ser/total-Ser (%)	D-Ala (μM)	D-Ala/total-Ala (%)	Cr (mg/dl)	D-Ser/Cr (nmol/mg)	L-Ser/Cr (nmol/mg)	D-Ala/Cr (nmol/mg)
A, male, 25 years old weight, 75 kg	19:30	123	127	49.2	39	26.2	165	75	77	24
	22:30	127	130	49.3	12	14.6	159	80	82	8
	1:00 (next day)	126	111	53.1	22	18.8	158	80	70	14
	9:00	368	146	71.6	62	24.3	386	95	38	16
	11:00	148	80	64.9	21	16.2	184	80	43	11
	14:00	92	85	52.1	21	19.3	124	74	69	17
	17:00	113	89	55.7	23	5.9	172	66	52	13
	20:40	163	127	56.1	31	12.9	228	71	56	14
	Average	158	112	56.5	29	17.3	197	78	61	15
	S.D.	88	25	7.9	16	6.5	82	9	16	5
	CV (%)	56	22	14.0	54	37.4	42	11	26	32
B, male, 33 years old weight, 60 kg	17:00	246	384	39.0	83	12.7	227	108	169	37
	22:10	182	292	38.4	57	12.4	182	100	160	31
	23:10	83	214	28.0	43	12.5	81	102	264	53
	8:00 (next day)	97	186	34.2	44	21.4	81	120	230	54
	12:40	214	324	39.8	101	12.6	153	140	212	66
	14:30	241	365	39.8	120	23.1	201	120	182	60
	Average	177	294	36.5	75	15.8	154	115	203	50
	S.D.	71	80	4.7	32	5.0	62	15	40	13
	CV (%)	40	27	12.8	43	31.9	40	13	20	27
C, female, 32 years old weight, 50 kg	6:45	174	391	30.8	51	14.8	136	128	288	38
	12:10	120	196	38.0	16	10.1	88	136	223	18
	17:20	102	167	38.0	15	8.1	128	80	130	12
	23:20	215	411	34.3	41	8.3	188	114	219	22
	6:25 (next day)	180	572	23.9	61	15.1	147	122	389	41
	8:45	55	127	30.8	15	13.8	46	120	276	33
	Average	141	311	32.6	33	11.7	122	117	254	27
	S.D.	59	175	5.4	21	3.2	49	20	86	12
	CV (%)	42	56	16.4	62	27.7	40	17	34	43

The three volunteers were leading a normal life and freely taking food and water.

Comparison of Urinary D-Ser Concentrations in Adult Human, Rat and Dog

Since D-Ser causes acute necrosis of the proximal tubule when administered to rats at a high dose,¹⁸⁻²¹⁾ we examined the D-Ser level in rat urine. As shown in Fig. 3, the D-Ser concentration in adult rat urine was much lower than that in human urine. In contrast, a high concentration of D-Ser was observed in adult dog urine. The D-Ser/total-Ser ratio was about 62%. In addition, a high D-Ala concentration has also been found in adult dog urine. D-Ala/total-Ala was about 67%.

Developmental Changes in D-Ser Excretion in Rat Urine

To investigate why the D-Ser excretion is low in the adult rat as compared to that in human and dog, we determined the urinary D-Ser in young rats since the activity of D-amino acid oxidase, an enzyme in the kidney involved in D-amino acids metabolism, was very low in young rats.^{14,15,25)} As shown in Fig. 4, there are great developmental changes in D-Ser and D-Ala excretion in rat urine. The D-Ser concentration in urine was high in young rats at the suckling (2 weeks) and after the weaned periods (3 weeks), and then declined with increasing age. In contrast, the urinary D-Ala concentration was very low in suckling rats and increased rapidly after the weaned state, then declined with the increasing age. The urinary D/D+L ratios of Ser and Ala also showed different patterns during growth. The D-Ser/total-Ser value was the highest at 2 weeks, whereas the D-Ala/total-Ala value was maximal at 3 weeks.

D-Ser in Plasma (or Serum) from Human, Rat or Dog

One possible factor that controls the urinary excretion of D-Ser may be the renal filtered load. We therefore compared the

Fig. 3. Species Differences of Urinary Excretion of D-Ser and D-Ala in Adult Human, Rat and Dog

Human data were from 18–55-year-old subjects ($n=11$). Nine-week-old male Wistar rats ($n=6$) were used for comparison. Dog data were from 2 male adult Beagle dogs.

D-Ser and D-Ala concentrations in their plasmas. As shown in Table 3, D-Ser and D-Ala concentrations in plasma were very low compared with those in urine. The difference of D/L ratio between urine and plasma may result from the stereoselective reabsorption of amino acids in the proximal tubule.^{26,27)} There were no significant differences in D-Ser concentrations among human, rat and dog serum (or plasma). Plasma L-Ser

Fig. 4. Developmental Changes in Urinary Excretion of D-Ser (A) and D-Ala (B) in Rats

Each value is the mean $\pm$ S.D. of the data from 3—6 animals. * $p < 0.01$ as compared with 3-week-old rats.

Table 3. D-Ser and D-Ala Concentrations in Plasma or Serum from Rat, Dog and Human

Species	Plasma or serum	Age	Sample numbers	D-Ser (μ M)	Total-Ser (μ M)	D-Ser/total-Ser (%)	D-Ala (μ M)	Total-Ala (μ M)	D-Ala/total-Ala (%)
Rat	Plasma	2 weeks	$n=5$	1.2 ± 0.4	186 ± 19	0.7 ± 0.2	$0.8 \pm 0.2^*$	253 ± 16	$0.3 \pm 0.1^*$
		3 weeks	$n=3$	1.0 ± 0.3	120 ± 25	0.9 ± 0.4	3.1 ± 1.2	309 ± 73	1.0 ± 0.5
		4 weeks	$n=5$	0.7 ± 0.2	134 ± 35	0.5 ± 0.2	8.3 ± 5.0	402 ± 112	1.9 ± 0.8
		6 weeks	$n=4$	1.9 ± 1.3	148 ± 40	1.3 ± 0.7	3.0 ± 1.7	332 ± 33	0.9 ± 0.5
		9 weeks	$n=3$	1.4 ± 1.8	92 ± 14	1.5 ± 0.9	3.2 ± 1.6	217 ± 27	1.5 ± 0.8
Dog ^{a)}	Plasma	Adult	$n=6$	1.3 ± 0.5	85 ± 22	1.5 ± 0.6	1.3 ± 1.1	294 ± 94	0.4 ± 0.3
Human ^{b)}	Serum	Adult	$n=10$	1.6 ± 0.5	115 ± 29	1.5 ± 0.4	1.0 ± 0.6	371 ± 87	0.3 ± 0.2

a) Dog blood samples were drawn from two dogs at 9:00, 17:00 and 1:00 h. b) Healthy human blood samples of 25- to 54-year-old individuals (male, 5; female, 5) were drawn in the morning after overnight fasting. Each value is expressed as the mean $\pm$ S.D. * $p < 0.01$ as compared with 3—9-week-old rats.

concentration in young rats was higher than that in old rats, D-Ser concentrations between young and old rats, however, were not so obviously different. In contrast, significantly lower plasma D-Ala concentrations were observed in 2-week-old rats than in 3—9-week-old rats. This indicated that the lower urinary D-Ala excretion in suckling rats resulted from their lower bloodstream supply. However, the low urinary D-Ala level in old rats was not due to their low filtered loads but to the filtered D-Ala from the bloodstream being metabolized by the kidney.

Free D-Ser and D-Ala Contents in Animal Foods and Human Colostrum One possible origin of urinary D-Ser was considered to be diet. To investigate this possibility, we determined the free D-Ser and D-Ala content in rat and dog diets and human colostrum. Free D-Ser and D-Ala were detected in each food as follows: rat food, D-Ser, 11 nmol/g; D/D+L, 1.7%; D-Ala, 135 nmol/g; D/D+L, 4.2%; dog food, D-Ser, 24 nmol/g; D/D+L, 2.1%; D-Ala, 382 nmol/g; D/D+L, 8.3%. Free D-Ser and D-Ala concentrations in human colostrum from 4 postpartum women were as follows: D-Ser, $0.4 \pm 0.2 \mu$ M; D/D+L, $2.2 \pm 1.1\%$; D-Ala, $0.3 \pm 0.1 \mu$ M; D/D+L, $1.2 \pm 0.8\%$ (mean $\pm$ S.D., $n=4$).

Recovery of D-Ser in Rat Urine after D-Ser Administration If urinary D-Ser was of food origin, D-Ser would be absorbed from the intestine. To prove this assumption, we compared urinary D-Ser concentrations before and after D-Ser was orally administrated to rats (10 mg/kg). Indeed, urinary D-Ser concentration was increased about 2.5 times after dos-

ing, which indicated that D-Ser could be absorbed and excreted into urine. However, the urinary recovery of the dosed D-Ser was only $1.2 \pm 0.7\%$, $n=3$. This means that most of the administered D-Ser was metabolized by the body or was not absorbed completely.

DISCUSSION

In the present study, we investigated urinary D-Ser excretion in humans of various ages. For comparison, D-Ser excretion in the urine of rats and dogs was also studied. Our results were in agreement with those which appeared in another report,¹⁷⁾ indicating that a great amount of D-Ser was excreted in human urine. Furthermore, we have observed that the urinary D-Ser/Cr value was relatively constant in adult individuals. The constant excretion of D-Ser in human urine was confirmed by the consecutive 24 h urine analyses. It has also been demonstrated that the urinary excretion of D-Ser is species- and age-related.

The species- and age-related excretion of D-Ser appears not to be due to the difference of renal filtered load, because the plasma concentrations were not so different among the species examined. The major reason is therefore considered to be variations in renal handling of D-Ser among different species and different ages. D-Amino acid oxidase activity was very low in newborn mammals and increased during the development.²⁵⁾ The lower excretion of D-Ser in adult rats (more than 6 weeks) than in young rats (less than 4 weeks)

may be a consequence of the change in their D-amino acid oxidase activity. However, urinary D-Ser excretion cannot be completely explained by the D-amino acid oxidase activity in the kidney alone, because a greater relative quantity of kidney D-amino acid oxidase in dogs than in rats was reported,²⁰⁾ whereas the urinary D-Ser level in dogs was higher than that in rats. A more important factor for controlling D-amino acid excretion in urine seems to be the renal amino acid transport system in which D-amino acids are reabsorbed and regulated. The D-Ser reabsorption rate is conceivably higher in adult rat kidney than in human and dog kidneys. The higher nephrotoxicity of D-Ser in rats than in other animals such as dog during high dose D-Ser administration^{19,20)} may also support the assumption that D-Ser is more efficiently reabsorbed by rat proximal tubules.

In order to investigate the origin of D-Ser in urine, we analyzed the free D-Ser content in the animals' food. Though free D-Ser was detected in rat and dog foods, the amount was small compared with the calculated value expected to be excreted in urine. If a rat weighing 100 g takes about 10 g of food per day, the dietary intake of free D-Ser is about 110 nmol/d; however the amount of D-Ser excreted in rat urine was about 500 nmol/d. Because D-Ser recovery in rat urine within 24 h was very low after the oral administration of D-Ser (<2%), the free D-Ser amount in food was not enough to account for the amount of D-Ser excreted in rat urine. The absolute and relative level of urinary free D-Ser was still high in suckling rats who did not take exogenous food other than the milk of their mothers. Although we did not measure the D-amino acids in maternal rat milk, the free D-Ser concentration ($0.4 \pm 0.2 \mu\text{M}$) in human colostrum was very low. Csapó *et al.* also reported that the free D-Ser in raw cow milk was very low ($0\text{--}4 \mu\text{M}$), though considerable amounts of free D-Ala ($5.3\text{--}321.3 \mu\text{M}$) were detected.²⁸⁾ Apparently, free D-Ser in milk is quite insufficient to fulfill the urinary D-Ser in suckling immatures.

In contrast, the urinary D-Ala level in suckling rats was much lower than in weaned rats. The rapidly increased D-Ala excretion in urine during the weaning period might be due to the beginning intake of food that contained higher D-Ala than their mothers' milk. Another possible reason could be a change in their intestinal bacteria during the weaning state because bacteria contain large amounts of D-Ala. Konno *et al.* also reported that the urinary excretion of D-Ala in mice was reduced after antibiotic administration and that the urinary D-Ala level in germ-free mice was much lower than in conventional mice.²⁹⁾ A much lower urinary D-proline concentration in suckling rats than in weaned rats has also been observed by Armstrong *et al.*³⁰⁾ Therefore, the high concentration of D-Ser excreted in suckling rat urine suggests that the origin of urinary D-Ser is different from that of other urinary D-amino acids.

Though racemization of amino acids in proteins may occur during the processing of food,³¹⁾ this unlikely to be the major source of urinary D-Ser, because the urinary D-Ser level was still high in suckling rats who did not take in exogenous food other than the milk of their mothers. If urinary D-Ser was mainly of dietary origin, the other parts of D-Ser in milk, such as D-Ser containing protein must be considered. However, no naturally occurring D-Ser containing protein (or peptide) in the milk has been reported.

Our present results indicate that food is one of the sources of urinary D-Ser in mammals but is not likely the major source. D-Ser in mammalian urine may be mainly of endogenous origin. This view is strongly supported by the fact that a high urinary D-Ser level was found in a 4-d-old infant who did not take any food but was infused only with a solution of glucose and electrolytes after birth.

Quite recently, there have been three reports concerning brain D-Ser synthesis. One is that D-Ser could be synthesized from L-phospho-serine in rat brain synaptosomes.³²⁾ Another is that a glycine cleavage system may also be involved in brain D-Ser synthesis or regulation.³³⁾ The third study indicated that D-Ser is probably synthesized by a racemase.³⁴⁾ However, the exact mechanism of brain D-Ser biosynthesis is not clarified. The metabolic fate of D-Ser in the brain is also mystery, because D-Ser is localized in the forebrain areas whereas D-amino acid oxidase does not exist.⁶⁾ At present, we do not know if there is some connection between brain D-Ser and urinary D-Ser. Perhaps urinary D-Ser was partly of renal origin because a considerable free D-Ser level was also detected in the kidney of young rats.¹⁶⁾ Whether D-Ser plays some physiological role in regulating renal functions or is just a waste product or a metabolite of the body that is highly excreted in urine is not known at present. Further studies will be needed to elucidate where and how the urinary D-Ser is formed.

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REFERENCES AND NOTES

- 1) The author is a recipient of a foreign postdoctoral fellowship from the Japan Society for Promotion of Sciences.
- 2) Imai K., Fukushima T., Santa T., Homma H., Hamase K., Sakai K., Kato M., *Biomed. Chromatogr.*, **10**, 303—312 (1996).
- 3) Hashimoto A., Nishikawa T., Oka T., Takahashi K., *J. Neurochem.*, **60**, 783—786 (1993).
- 4) Hashimoto A., Kumashiro S., Nishikawa T., Oka T., Takahashi K., Mito T., Takashima S., Doi N., Mizutani Y., Yamazaki T., Kaneko T., Ootomo E., *J. Neurochem.*, **61**, 348—351 (1993).
- 5) Nagata Y., Horiike K., Maeda T., *Brain Res.*, **634**, 291—295 (1994).
- 6) Schell M. J., Molliver M. E., Snyder S. H., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 3948—3952 (1995).
- 7) Schell M. J., Brady R. O., Jr., Molliver M. E., Snyder S. H., *J. Neurosci.*, **17**, 1604—1615 (1997).
- 8) Danysz A., Fadda E., Wroblewski J. T., Costa E., *Life Sci.*, **46**, 155—164 (1990).
- 9) Salt T. E., *Brain Res.*, **481**, 403—406 (1989).
- 10) Chouinard M. L., Gaitan D., Wood P. L., *J. Neurochem.*, **61**, 1561—1564 (1993).
- 11) Nagata Y., Borghi M., Fisher G. H., D'aniello A., *Brain Res. Bull.*, **38**, 181—183 (1995).
- 12) Kumashiro S., Hashimoto A., Nishikawa T., *Brain Res.*, **681**, 117—125 (1995).
- 13) Nagata Y., Shoji R., Yonezawa S., Oda S., *Amino Acids*, **12**, 95—100 (1997).
- 14) Nagata Y., *Experientia*, **48**, 753—755 (1992).
- 15) Hashimoto A., Nishikawa T., Konno R., Niwa A., Yasumura Y., Oka

- T., Takahashi K., *Neurosci. Lett.*, **152**, 33—36 (1993).
- 16) Hashimoto A., Oka T., Nishikawa T., *Eur. J. Neurosci.*, **7**, 1657—1663 (1995).
- 17) Brückner H., Haasmann S., Friedrich A., *Amino Acids*, **6**, 205—211 (1994).
- 18) Morehead R. P., Poe W. D., Williams L. M., Lazenby M. E., *Am. J. Pathol.*, **22**, 658 (1946).
- 19) Ganote C. E., Peterson D. R., Carone F. A., *Am. J. Pathol.*, **77**, 269—282 (1974).
- 20) Kaltenbach J. P., Canote C. E., Carone F. A., *Exper. Mol. Pathol.*, **30**, 209—214 (1979).
- 21) Carone F. A., Nakamura S. and Goldman B., *Lab. Invest.*, **52**, 605—610 (1985).
- 22) Kotaniguchi H., Kawakatsu M., Toyooka T., Imai K., *J. Chromatogr.*, **420**, 141—145 (1987).
- 23) Fukushima T., Kato M., Santa T., Imai K., *Biomed. Chromatogr.*, **9**, 10—17 (1995).
- 24) Hamase K., Homma H., Takigawa Y., Fukushima T., Santa T., Imai K., *Biochim. Biophys. Acta*, **1334**, 214—222 (1997).
- 25) Meister A., Wellner D., Scott S., *J. Natl. Cancer Inst.*, **24**, 31—49 (1960).
- 26) Young J. A., Freedman B. S., *Clin. Chem.*, **17**, 245—266 (1971).
- 27) Silbernagl S., *Physiol. Rev.*, **68**, 911—1007 (1988).
- 28) Csapó J., Csapó-Kiss Z., Stefler J., Martin T. G., Némethy S., *J. Dairy Sci.*, **78**, 2375—2381 (1995).
- 29) Konno R., Oowada T., Ozaki A., Iida T., Niwa A., Yasumura Y., Mizutani T., *Am. J. Physiol.*, **265**, G699—G703 (1993).
- 30) Armstrong D. W., Gasper M. P., Lee S. H., Ercal N., Zukowski J., *Amino Acids*, **5**, 299—315 (1993).
- 31) Man E. H., Bada J. L., *Ann. Rev. Nutr.*, **7**, 209—225 (1987).
- 32) Wood P. L., Hawkinson J. E., Goodnough D. B., *J. Neurochem.*, **67**, 1485—1490 (1996).
- 33) Iwama H., Takahashi K., Kure S., Hayashi F., Narisawa K., Tada K., Mizoguchi M., Takashima S., Tomita U., Nishikawa T., *Biochem. Biophys. Res. Commun.*, **231**, 793—796 (1997).
- 34) Dunlop D. S. and Neidle A., *Biochem. Biophys. Res. Commun.*, **235**, 26—30 (1997).