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Factors controlling the level and determination of D-amino acids in the urine and plasma of laboratory rodents

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Summary. Unambiguous methodologies were developed for the accurate and reproducible determination of specific D-amino acids in the physiological fluids of common laboratory rodents. Depending on the strain of rodent and the type of amino acid examined, excreted D-amino acids ranged from the low percent levels to over 40 percent of the total specific amino acid level. Relative plasma levels tended to be considerably lower, typically an order of magnitude less. A number of factors were found to alter the relative amounts of excreted D-amino acids. This included: diet, age, pregnancy, advanced cancer, and antibiotics. The two factors that seemed to result in substantially lower levels of excreted D-amino acids were fasting and young age. Pregnancy was the only factor that consistently resulted in higher relative D-amino acid excretion. Much of the observed data are believed to be related to the efficiency with which the kidney reabsorbs L-amino acids. No claims are made as to the meaning and/or importance of free D-amino acids in regards to pathology, age, clinical usefulness and so forth. However, a knowledge of normal D-amino acid levels and dynamics is necessary before it is possible to identify perturbations caused by either natural or pathological conditions. The techniques are now available that should allow these topics to be addressed properly.

Keywords: D-amino acids – Urine – Plasma – Food – Excretion – Racemization – Kidney function.

Introduction

It is well known that the proteins of living organisms have twenty primary constituents (i.e., 18 L-α-amino acids, one cyclic L-α-imino acid, proline and one nonchiral α-amino acid, glycine). These primary constituents exist not

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only in proteins, but also as oligomers (peptides) and as free amino acids throughout all organisms (Hardy, 1985). In mammals it is generally believed that naturally occurring amino acids are "stereochemically pure" and of the L-configuration (Hoeprich, 1965). Although L-amino acids appear to predominate in all other living systems as well, there are a few well documented cases of the use of D-amino acids by certain microorganisms, particularly as structural elements of cell walls and in certain cyclic peptides (Schleifer and Kandler, 1972; Wheat, 1988).

Recently, reports have appeared which indicate that finite levels of D-amino acids exist in mammals, i.e., humans and laboratory rodents (Armstrong et al., 1991; Nagata et al., 1987; Konno et al., 1989; Konno et al., 1988a; Nagata et al., 1989; Fisher et al., 1986) as well as marine invertebrates (Preston, 1987, 1990). However, there is considerable controversy over their origin, level and biological role, if any (Konno et al., 1990). For example, evidence has been presented that D-amino acids in mammals may (or may not) derive from the degradation of proteins, associated bacteria, food sources and so forth (Nagata and Akino, 1990). The fact that there is some controversy in this area should not be surprising considering the accuracy of some past reports and the sometimes difficult methodologies involved (Konno et al., 1988b; Konno et al., 1990; Kogl, 1949). For example, over 50 years ago it was reported that hydrosylates of tumor proteins contained excess D-amino acids (Kögl and Erxleben, 1939). Although some controversy continued for over a decade, most now believe that the presence of any D-amino acids in these studies resulted from the acid hydrolysis step and/or other means (Kögl, 1949; Miller, 1950). More recently, the apparent accumulation of D-aspartic acid in the human brain with age (Man et al., 1983) was shown not to be reliable because of accuracy and reproducibility problems in the analytical methodologies employed (Payan et al., 1985). On the other hand the accumulation of D-aspartic acid with time in teeth (Helfman and Bada, 1975) and the eye lens (Masters et al., 1977) seems to be reasonably well documented and reproducible. However, in these particular structures, the proteins (once formed) are relatively isolated from the circulatory system and therefore are not continually degraded and regenerated as are most other proteins. Hence the accumulation of D-aspartic acid (one of the most rapidly racemized amino acids) is thought to occur via slow racemization of the originally deposited L-enantiomers.

The existence of the enzyme D-amino acid oxidase in the organs and brains of most higher animals is well documented (Konno et al., 1982). This enzyme catalyzes the oxidative degradation of several, but not all, D-amino acids (Krebs, 1935). The necessity and physiological role of this enzyme is not well understood (Moreno, 1986). At least one mutant strain of mouse has been bred which lacks D-amino acid oxidase (Konno and Yasumura, 1983). Whether or not this strain of rodent suffered from any adverse effects resulting from this deficiency was not mentioned. Also there has been at least one report where a pathologic syndrome was associated with the reduced activity of several oxidative enzymes, one of which happened to be D-amino acid oxidase (Goldfischer et al., 1986). Finally, it was reported that elevated levels of D-amino acids were found associated with kidney diseases and aging in humans (Nagata et al., 1987). However, it should

be noted that this was not a specific determination for individual amino acids but rather a general enzymatic method (employing D-amino acid oxidase) that produced a combined number for all substrates to that enzyme (Nagata et al., 1987).

Given the importance of amino acids in all living systems and their association with and use in the diagnosis of certain diseases, it is reasonable to examine the occurrence and disposition of free D-amino acids. However, the procedures and methodologies must be available that provide reproducible and unambiguous results. We have developed two such methodologies and have used them to examine the D-amino content in the urine and plasma of eight common laboratory rodents. The factors affecting D-amino acid content are examined. Based on current results as well as past data we categorize what, in our opinion, can and cannot be said about D-amino acids in mammals.

Materials and methods

Chemicals

Amino acids were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile, water, acetic acid and triethylamine were of Omnisolve grade and supplied by EM Science (Gibbstown, NJ). The derivatizing agent, 9-fluorenyl methylchloroformate, was purchased from Sigma (St. Louis, MO). The perchloric acid, mercaptoethanol, boric acid, o-phthalic dicarboxaldehyde (OPA), and DL-amino acids were obtained from Aldrich (Milwaukee, WI). Methanol, ethanol and potassium hydroxide were supplied by Fisher (St. Louis, MO).

Animal preparation

Rodents were obtained from Harlan Sprague Dawley, Inc., in Indianapolis and were acclimatized at least 1 week in the Animal Care Facility. During that time, they were allowed ad libitum of standard rat chow(Purina 5001, Montgomery, MO) and water, at 24°C with a 12 hour light/dark cycle, unless otherwise indicated. Urine samples were collected carefully in the mornings, derivatized in some cases and analyzed immediately. Rapid derivatization and/or analysis of these samples is imperative.

Plasma samples

Citrated blood (1 ml of 3.8% Na citrate:9 ml blood) was centrifuged for 5 minutes to obtain plasma for D-amino acid measurements.

Diet study

C57BL, DBA and Sprague-Dawley rodents were given only 5% glucose in water for 72 hrs. Urine D-Phe and D-Pro concentrations only were determined because during fasting, smaller quantities of urine were excreted.

Antibiotic study

C57BL and C3H (8 animals for each) were given 0.02 % Amoxicillin + 1% glucose or 0.02% Neomycin sulfate + 1% glucose in water. The water, containing antibiotics and glucose, was changed every day.

Pregnancy and age studies

Urine samples were collected from 2–3 weeks pregnant mice and analyzed by the procedure outlined below. In a separate study, urine samples were obtained from a breast-fed litter of 8 C57BL mice and 4 Sprague Dawley rats (all from one litter). These samples were analyzed for D-amino acid content and compared to mature rodents of the same species as well as to juvenile rodents of the same age that were separated from their mother.

Advanced tumor study

In order to generate tumor 2.10^5, Lewis Lung Carcinoma cells in 0.2 ml of media was injected into gastrocnemius muscle. Blood and urine samples were obtained between 25 and 30 days later. These rodents were all part of a separate unrelated project. The urine samples not needed for the primary project were donated to this study.

D-Proline analysis

Derivatization was performed according to Einarsson et al., 1983. After derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl), 5–50 μL of the sample was injected into a C_{18} reversed phase column (100 × 4.6 mm) supplied by Advanced Separation Technology (Whippany, NJ). The mobile phase consisted of acetonitrile + water + acetic acid (380 + 620 + 2, v/v). The flow rate was 0.5 ml/min. A UV wavelength of 266 nm was used to monitor the effluent. The column switching value was turned for 2–15 seconds after the signal reached the maximum of the standard retention time. A small portion of the eluting peak of FMOC-proline was introduced into the RN-β-CD chiral columns (100 × 4.6 mm) from Advanced Separation Technology (Whippany, NJ) and eluted with acetonitrile + acetic acid eluent (1000 + 6, v/v) at 1 ml/min. The effluent from chiral column was monitored using fluorescence detection. Figure 1 shows both the achiral (C_{18}) and chiral (CB-I-RN) separation of FMOC-proline from the urine of a C3H mouse. It is important to note that in proper aqueous buffer, no detectable racemization of the proline occurs during the derivatization process (Zukowski et al., 1992). Furthermore, once the FMOC derivative is formed, no change in enantiomeric ratios were found even after several weeks.

D-Phenylalanine, D-tyrosine, D-tryptophan, D-leucine analyses

The LC system which utilized post-column fluorescence detection was made up of the following Shimadzu devices: two LC-6A pumps, a SCL-6B system controller, a CR601 chromatopac recorder, SPD-6AV UV/VIS spectrophotometric detector and a RF-535 fluorescence HPLC monitor. The "fluorescence" chromatographic system also included a Rainin (San Carlos, CA) model A-30-S pump, Scientific Systems, Inc. (State College, PA), pulse dampener model LP-21 and two Rheodyne injectors (models 7125 and 7010) for post-column derivatization.

The chromatographic columns used in these studies included the Crownpak CR(+) and Crownpak CR(−) supplied by Michael Henry at J.T. Baker (Phillipsburg, NJ). Crownpak columns used for post-derivatization were used at 5°C. All the samples and mobile phase solutions were filtered using 0.2 μm filters supplied by Alltech Associates, Inc. (Deerfield, IL).

A C_{18}column was used to isolate the individual amino acids. The mobile phase consisted of 95:5, water: methanol (v:v). The flow rate was 1 or 1.5 ml/min. The column was at room temperature and the UV detector setting was at 195 nm. A column switching or coupled column method was used for the analysis of tyrosine, phenylalanine, tryptophan in rat or mouse urine. After separation on the C_{18} column, individual amino acids were switched onto the chiral crown ether column in order to determine their enantiomeric ratio.

Post column OPA derivatization of the amino acids was required for fluorescence detection. The excitation wavelength was 340 nm and the emission wavelength was 450 nm. The Rainin pump was the post column pump for this system. The OPA solution was

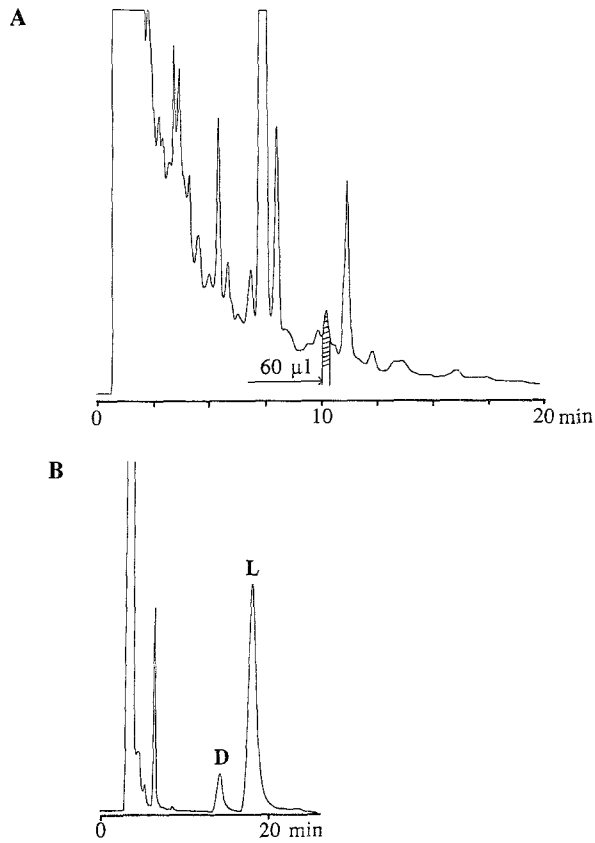

Fig. 1. Chromatogram **A** shows the C_{18}-reversed phase separation of FMOC derivatized amino acids from the urine of a male C3H mouse. The "cross-hatched-peak" is FMOC-proline which was switched onto the chiral Cyclobond I-RN column in order to determine the enantiomeric ratio (chromatogram **B**). This particular sample was about 12% D-proline. The reversed phase column (100 × 4.6 mm i.d.) contained 3μ support. The mobile phase consisted of 41:59:02, by volume, acetonitrile:water:acetic acid. The flow rate was 0.5 ml/min and the UV detection wavelength was 266 nm. The chiral column (250 × 4.6 mm, i.d.) contained 5μ support. The mobile phase consisted of 100:0.6, by volume, acetonitrile: acetic acid. The flow rate was 1.0 ml/min. Fluorimetric detection was used λex = 266 nm, λem = 315 nm)

prepared as follows: dissolve 700 mg of o-phthalic dicarboxaldehyde in 15 ml of ethanol. Three hundred microliters of mercaptoethanol was added to this solution. The solution was then added to 1 liter of 3% boric acid solution which had been adjusted to pH 10.0 with KOH. Teflon tubing (0.5 mm id) was used with the post column apparatus and the post column reactor was 3 m long. In order to further verify that no racemization occurred during the post-column method, the FMOC-gly-Cl precolumn derivatization method was also used. Because FMOC-gly-Cl is not commercially available, it was synthesized in our laboratory. Derivatization was performed according to Einarsson et al. (1983). The procedure for the precolumn FMOC-gly-Cl method was essentially the same as the described for FMOC-proline above. In order for any results to be considered valid, the same answer had to be obtained (within experimental error) by each of the two methods (i.e., precolumn and post-column). In addition the opposite chirality crown ether column was used to reverse the retention order of the amino acid enantiomers of interest. Figure 2 shows the enantiomeric separation of D- and L-phenylalanine from DBA mouse urine. In order to further verify this

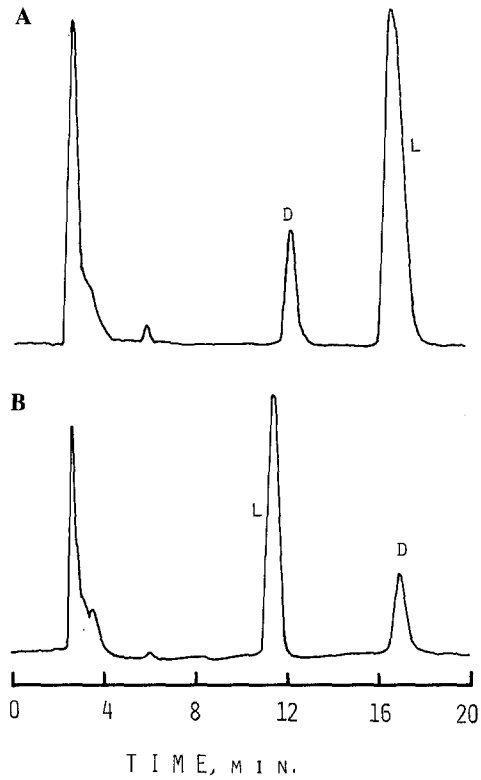

Fig. 2. Chromatogram **A** shows the enantiomeric separation of D and L-phenylalanine on a CR(+) chiral crown ether column. Chromatogram **B** shows the same sample on the opposite configuration CR(−) column. This sample was from the urine of a DBA mouse which was directly injected onto a C_{18} column. Subsequently the eluted phenylalanine peak was switched onto the aforementioned chiral crown ether columns. Approximately 18% of the total phenylalanine was the D-enantiomer. Experimental conditions are given in the Materials and methods section

separation, the sample was divided and run on chiral columns of the opposite configuration. This reverses the retention order of the phenylalanine enantiomers but does not affect the retention of any achiral impurities. Water extracts of the animal feed were analyzed by both post-column and precolumn methods. As in all of the analyses in this work, only free amino acids were measured. This does not take into account the possibility that proteins in the feed (having been subjected to "unusual" conditions during processing) contain small quantities of D-amino acids which can be released upon digestion.

In all measurements done on biological samples (vide supra) care must be taken to prevent chemical racemization and/or microbiological contamination. The procedures outlined in this section have been shown to produce little or no chemical racemization down to the parts per ten thousand level or lower levels in some cases (Armstrong et al., 1991; Zukowski et al., 1992). More often microbiological contamination of a sample can be a problem. Contamination can cause an increase or decrease in relative, D-amino acid levels (depending on the type of sample and length or type of microbial contamination. In some cases the level of both D and L amino acids decrease dramatically. Only sterile samples frozen immediately after collection could be stored for latter analysis. In this study all underivatized samples were analyzed immediately after collection. When derivatizing amino acids, the reaction was done at the animal care facility immediately upon collection of the sample.

Results

Excretion of the amino acids phenylalanine, tyrosine, tryptophan, leucine and proline was examined in eight types of common laboratory rodents (i.e., four different strains of mice and four strains of rats). All specimens were provided with identical diets (see Materials and methods). The results are summarized in Table 1. The level of total amino acid excretion seems to be consistent with previously reported results (Konno et al., 1988b; Rivera et al., 1987). Interestingly, a relatively high percentage of the excreted amino acids are of the D-configuration. D-amino acid levels in the 10 to 20% range are common and even higher levels (>30 to 40%, see D-proline in the C57BL mouse, Table 1) are frequently found. The level of D-amino acid output in most laboratory rodents seems to be significantly higher than that reported for humans (Armstrong, 1991). Also, the variation in the D-amino acid levels in rodents seemed to be far less than that found for humans. However, this last trend is likely due to the fact that rodents were in a controlled environment whereas the humans were not. Two other things are evident from the data in Table 1. First, different strains of rodents consistently excrete different relative levels of D-amino acids. For example the Balb/c mice generally excreted lower relative levels of all D-amino acids, while C57BL mice generally excreted higher relative levels (Table 1). Finally, within any one strain of rodent, both the total level of excreted amino acid and the % D-amino acid excreted vary considerably with the type of amino acid examined (Table 1)

Several studies were done in order to identify factors that affect D-amino acid excretion in rodents. Table 2 summarizes the results of limiting food intake on D-amino acid output. Over the course of 3 days the rodents were allowed free access to an aqueous glucose solution, but no other food. Clearly the relative levels of D-amino acids fell precipitously the first 24 to 48 hours and then stabilized at levels between about 1 to 4% depending on the strain of rodent examined. Interestingly, the total amino acid output did not decrease as precipitously as one might expect. The continued excretion of significant levels of amino acids during "starvation" has been described before (Konno et al., 1990).

In an effort to study the possible effect of gut bacteria on D-amino acid output, three different strains of rodents (two mice and one rat) were given antibiotics and their excretion of D-proline and D-phenylalanine was monitored. The antibiotics were dissolved in the drinking water along with 1% glucose to help mask taste differences (Material and methods). The results are shown in Table 3. Two different antibiotics were used in separate experiments. As can be seen in Table 3, both antibiotics affected the relative amount of excreted D-amino acids, but in a somewhat "chaotic" manner. In contrast with the previous study where food was withheld (Table 2) there was no consistent decrease in the relative amounts of excreted D-amino acids. In fact different effects were sometimes observed for the two antibiotics. Further complicating the situation was the fact that the two amino acids monitored did not always "behave" in an analogous manner. For example, after the rodents had been treated with neomycin for 48 and 72 hours, the amount of excreted D-proline

Table 1. Relative D-amino acid output in urine of mature laboratory rodents[a]

Total	Phenylalanine		Tyrosine		Tryptophan		Leucine		Proline	
	% D[b]	Total Phe[c] μm/L	% D[b]	Total Tyr[c] μm/L	% D[b]	Total Trp[c] μm/L	% D[b]	Total Leu[c] μm/L	% D[b]	Total Pro[c] μm/L
Mouse										
1) C57BL/6 (female and male)	28 (4)	260	22 (4)	120	22 (4)	110	7 (2)	—	43 (5)	32
2) BALB/c	10 (2)	280	11 (2)	115	7 (2)	83	0.5 (0.3)	—	12 (2)	93
3) C3H/HeNHsd (female)	26 (5)	235	32 (5)	163	21 (5)	101	—	—	11 (3)	24
4) DBA/2NHsd (female)	20 (4)	370	12 (2)	60	30 (4)	68	—	—	10 (2)	112
Rat										
1) Sprague-Dawley	18 (5)	310	10 (4)	149	12 (3)	30	3 (1)	—	9 (2)	48
2) Fischer (F344/NHsd) (female)	36 (7)	350	11 (2)	107	23 (5)	80	—	—	5 (1)	19
3) ACI/seq Hsd (male)	23 (3)	355	19 (3)	80	17 (2)	87	—	—	5 (1)	92
4) Lewis (Lew/SsNHsd) (female)	14 (3)	265	20 (4)	118	21 (3)	38	—	—	12 (2)	108

[a] All animals were >3 months old before testing. No fewer than four rodents of each strain were tested

[b] The number shown is the mean of a minimum of three separate analyses performed on at least four different rodents and is rounded to the nearest percent. The number in parenthesis is the average deviation from the mean

[c] This number represents the total amino acid excreted regardless of chirality (i.e., L plus D enantiomers) and is the average of three determinations

Table 2. Effect of limited food intake on average D-amino acid output in urine[a]

Rodent	Amino acid: Phenylalanine % D[b]	Phenylalanine Total Phe[c] μm/L	Proline % D[b]	Proline Total Pro[c] μm/L
1) C57BL				
Control	28 (4)	260	43 (5)	32
24 hours	10 (2)		1 (0.2)	20
48 hours	7 (1)		2 (0.2)	15
72 hours	—		2 (0.3)	45
2) DBA/2NHsd				
Control	20 (4)	370	10 (2)	112
24 hours	14 (3)		6 (0.1)	80
48 hours	8 (2)		1 (0.2)	53
72 hours	—		2 (0.3)	71
3) Sprague-Dawley				
Control	18 (5)	310	9.(2)	48
24 hours	16 (4)		3 (0.5)	31
48 hours	2 (1)		4 (0.5)	23
72 hours	3 (1)		—	—

[a] Each strain of rodent was placed is isolated cages and fed only a 5% glucose solution. If any specimen appeared to be overly lethargic or suffering in any way they were removed from the experiment and returned to a normal diet. Six rodents of each strain were used in this study

[b] All of these are mean values and are rounded to the nearest percent. The number is parenthesis is the average deviation from the mean. Using the optimum procedures outlined in the Materials and methods section, 0.02% D-phenylalanine can be detected in the presence of 99.98% of the L-enantiomer. As little as 0.001% of D-proline can be detected in the presence of 99.999% of the L-enantiomer

c This number represents the total amino acid excreted regardless of chirality (i.e., L plus D enantiomers) and is the average of three determinations

increased above control levels after which it fell to control or below control levels. When the same experiment was run using amoxicillin, each rodent strain showed a different excretion behavior for D-proline (Table 3). The D-proline excretion by C57BL mice seemed to be unaffected by amoxicillin while the C3H mice showed an initial decrease in D-proline levels, followed by an increase by day 7. Conversely the Sprague Dawley Rats consistently excreted higher levels of D-proline during the entire "amoxicillin experiment". The excretion of D-phenylalanine showed similar inconsistent trends (Table 3). There is little doubt that antibiotics seem to affect the relative output of D-amino acids in these rodents (Except perhaps in the amoxicillin/C57BL case). However, there was no consistent increase or decrease observed with time, and there was certainly no consistent and dramatic lowering of the relative D-amino acid levels as was

Table 3. Effect of the antibiotic amoxicillin and neomycin sulfate on D-amino acid output in urine[a]

	Amoxicillin				Neomycin sulfate			
	Phenylalanine		Proline		Phenylalanine		Proline	
	% D[b]	Total Phe[c] μm/L	% D[b]	Total Pro[c] μm/L	% D[b]	Total Phe[c] μm/L	% D[b]	Total Pro[c] μm/L
1) C57BL								
Control	28 (4)	260	43 (5)	32	28 (4)	260	43 (5)	32
2 days	—		41 (5)	—	15 (3)		60 (8)	—
3 days	10 (2)		36 (5)	40	25 (5)		56 (7)	17
7 days	21 (3)		43 (6)	60	27 (5)		22 (4)	45
2) C3H								
Control	26 (5)	235	11 (3)	24	26 (5)	235	11 (3)	24
2 days	—		5 (2)	—	14 (3)		43 (6)	
3 days	20 (4)		4 (1)	10	—		49 (6)	48
7 days	43 (7)		27 (5)	53	23 (5)		6 (2)	31
3) Sprange-Dawley								
Control	12 (3)	310	9 (2)	48	12 (3)	310	9 (2)	48
2 days	9 (2)		23 (4)	28	—		15 (3)	—
3 days	10 (3)		24 (4)	41	7 (2)		22 (4)	31
7 days	11 (3)		27 (5)	18	—		9 (3)	15

[a] The conditions of this study are given in the Materials and methods section. Six rodents of each strain were used in this study

[b] The number shown is the mean and the adjacent number in parenthesis is the average deviation from the mean

[c] This number represents the total amino acid excreted regardless of chirality (i.e., L plus D enantiomers) and is the average of three determinations

observed in the aforementioned fasting experiment. It is clear that additional well designed and tightly controlled experiments must be done before the effects of antibiotics on amino acid excretion is understood. Also the fact that some antibiotics may contain D-amino acid moieties must be considered.

Four other factors were found to affect D-amino acid excretion in laboratory rodents. These include: age, pregnancy, advanced cancer and in some strains, sex. All of the factors that were found to affect D-amino acid output are summarized in Table 4. Newborn and very young rodents consistently excreted much lower levels of all D-amino acids tested. In some cases the level of D-amino acid excretion appeared to gradually increase with age until maturity. In mature, adult rodents the level of D-amino acid concentration remains relatively constant (under controlled conditions) unless it is perturbed by one of the other factors listed in Table 4. As shown in Tables 3 and 2, starvation (or possibly other dietetic changes) was the only factor found that consistently lowered D-amino acid excretion to near juvenile levels. Rodents in the advanced stages of cancer also showed lower levels of relative D-amino acid excretion Table 4. However, it should be noted that this may not be a direct result of the disease, but might be a secondary effect resulting from anorexia (see Discussion).

One thing that appeared to produce a consistent increase in D-amino acid excretion (at least in the case of proline) was pregnancy (Table 4). This trend

Table 4. Summary of factors other than "type" or strain of laboratory rodent that affect the excretion of D-proline

	% D-Proline in control rodent[a]	% D-Proline in test rodent[a]	Δ % D[b]
1) Age			
C57BL	43 (5)	1 (0.5)(14 days old)[c]	−98%
Sprague-Dawley	9 (2)	1 (0.4)(14 days old)[d]	−89%
Sprague-Dawley	9 (2)	5 (1)(31 days old)[d]	−44%
2) Pregnancy			
C3H	11 (3)	26 (4)[e]	+136%
DBA	10 (2)	22 (2)[e]	+120%
3) Fasting (Diet)			
C57BL	43 (5)	2 (0.3)(72 hours)[f]	−95%
DBA	10 (2)	2 (0.2)(72 hours)[f]	−80%
4) Advanced tumorigenesis			
C57BL	43 (5)	4 (1)[g]	−91%
5) Sex			
C3H	34 (7) (male)	11 (3) (female)[h]	−65%
ACI	5 (1) (male)	9 (2) (female)[h]	+80%
C57BL	43 (4) (male)	44 (5) (female)[h]	~0%

[a] All mean values are rounded to the nearest whole percent. The adjoining number is parenthesis is the average deviation from the mean

[b] These numbers represent the % increase or decrease in relative D-proline concentrations of the test rodent (as compared to the control rodent). They were calculated using the formula: $\Delta\%D = [(\%D_{test}) - (\%D_{control})] \times 100/(\%D_{control})$

[c] Eight rodents tested

[d] Four rodents tested

[e] Three rodents tested

[f] See Table II for complete results

[g] This decrease was only noted in the final stages of this disease (after day 25). As indicated in the text, this decrease is probably related to the fact that the test rodents eat very little at this point

[h] Four rodents tested

was particularly easy to see in those rodents that normally excreted lower levels of D-amino acids (<20%). It was not unusual to see a two to three-fold increase in D-amino acid excretion during pregnancy. Possible reasons for this will be considered in the following Discussion section.

In some but not all cases, there appeared to be a sex related difference in the D-amino acid excretion of rodents (see Table 4). For example, the average relative amount of D-proline excreted by C3H male mice was almost three times that of their C3H female counterparts. Conversely, ACI female rats tended to excrete greater percentages of D-proline than the males (Table 4). On the other hand, C57BL male and female mice appeared to excrete about the same percent D-proline within experimental error. It is clear from these results that in some, but not all rodent strains, there may be significant differences in amino acid metabolism and elimination between males and females. Hence in any study of

this sort, all of these variables must be monitored, controlled and reported if the experimental results are to be interpreted correctly. Also, as shown in the antibiotic experiment (Table 3) the behavior of one amino acid can be different or opposite to that of another.

Clearly, the two factors that consistently correlated with a pronounced decrease in relative D-amino acid excretion were diet and very young age. The main factor found that consistently produced an increase in the relative level of D-amino acid excretion was pregnancy.

Table 5 gives the total plasma level of free phenylalanine and proline and the percent of each D-amino acid present in several different rodents. A comparison of the relative amounts of D-amino acids in plasma versus urine (Table 1) for the same strain indicates that the relative plasma levels are generally an order of magnitude less than the relative urine levels. Analogous results were found in humans (Armstrong et al., 1991).

Table 5. Average plasma levels of D-amino acids in mature laboratory rodents[a]

	Proline		**Phenylalanine**	
	% D[b]	Total Pro[c] μm/L	% D[b]	Total Phe[c] μm/L
Mouse				
1) C57BL/6	4.3 (0.3)	85	0.1 (0.03)	25
2) BALB/c	1.2 (0.05)	98	—	—
Rat				
1) Sprague-Dawley	0.2 (0.05)	49	—	—
2) Fisher (F344/NHsd)	0.2 (0.07)	105	0.6 (0.1)	18
3) ACI/seq Hsd	0.4 (0.1)	95	—	—
4) Lewis (Lew/SsNHsd)	0.3 (0.1)	48	0.7 (0.2)	25

[a] All animals were > 3 months old before testing. Samples were taken from four different rodents of each strain

[b] The number reported is the mean and the adjacent number in paranthesis is the average deviation from the mean

[c] This number represents the total amino acid excreted regardless of chirality (i.e., L plus D enantiomers) and is the average of three determinations

Discussion

It is clear from the results that all rodents tested have significant levels of D-amino acids in their urine and plasma. In general, the relative plasma levels of D-amino acids were about ten times lower than analogous urine levels (Table 1 and Table 5). The relatively high levels of these apparently nonessential D-amino acids were unexpected. For some D-amino acids, urine levels above 30 to 40% were not uncommon. However these levels varied considerably for different amino acids and different strains of rodents. (Table 1).

It is well known that L-amino acids are reabsorbed by the proximal tubules of the mammalian kidney as they are useful entities (Berne and Levy, 1988). Therein probably is the reason that D-amino acid excretion levels seem relatively

high. If L-amino acids are actively and relatively efficiently retained (recycled) but D-amino acids are not, one would expect to find the proportion of D-amino acids in the urine to be high (or at least higher than in plasma). Mammalian kidneys and other organs also contain D-amino acid oxidase (Momoi, et al., 1988). While this study did not focus on the role or activity of this enzyme, it is apparent that significant quantities of D-amino acids seem to pass through the kidney, intact. A previous study on a mutant strain of mouse that lacked D-amino acid oxidase reported even higher levels of D-amino acid excretion (Konno et al., 1989).

The results in Table 4 clearly indicate than many different factors can effect the relative level of D-amino acids in physiological fluids. One of the more interesting of these factors is that of age. Specifically, newborn and young rodents almost always seem to have substantially lower percentages of D-amino acids in their urine and serum. Equally interesting is the fact that this level seems to increase as the animal grows. Sometime before maturity the relative D-amino acid content of these fluids either "stabilizes" or increases much more slowly. The term "stabilizes" should be qualified in the context of this work. Even in mature rodents, daily fluctuations in both the total amino acid content and % D-amino acid levels are observed (Table 1). Obviously any of the other factors listed in Table 4 can cause more drastic upward or downward fluctuations. However, in a tightly controlled environment and in the absence mitigating effects, the % D-amino acids in mature rodents seemed to remain relatively constant (e.g., relatively small fluctuations in either direction) whereas in very young rodents the % proline and phenylalanine increased with time.

Because of the apparent effect of diet on D-amino acid excretion, it was decided to repeat the aforementioned "rodent age study". In this case the young rodents were separated from their mothers after 15–21 days and placed on an adult diet (see Materials and methods). In these cases the relative level of D-amino acid excretion rapidly increased to adult levels. Apparently the earlier, more gradual increase in relative D-amino acid excretion with age was largely a reflection of the change in diet of young rodents (as they progressed from nursing to weaned states).

It is possible that the lower relative levels of D-amino acid excretion in rodents with advanced cancer are diet related as well (Table 4). It was consistently noted that the C57BL mice consumed far less food in the final disease stages. Hence one would expect a lowering of the relative D-amino acid output as was observed in the study where food intake was limited (Table 2).

There has been at least one report where extreme age in humans was reported to coincide with a higher than normal D-amino acid content in plasma (Nagata et al., 1987). However, this earlier study did not focus on any particular amino acid but rather used a general enzymatic method that produces a single number for all substrates to D-amino acid oxidase. Several of the rodents in the present study continue to be monitored to see if further increases in the absolute and relative amounts of D-amino acids occur with age. As yet nothing notable has been detected.

As a result of the age and diet studies the food supply of the laboratory rodent's was analyzed for free D-amino acids. The results are shown in Table

Table 6. Mean levels of free D-amino acids in rodent food[a]

Phenylalanine % D[b]	Tyrosine % D[c]	Tryptophan % D[d]	Proline % D[e]
2.2 (1.0–5.1)	3.7 (0.3–8.4)	4.2 (1.8–8.6)	1.0 (0.5–2.0)

[a] The dry food samples were ground, extracted with distilled water, filtered and analyzed immediately. The mean value is the first number shown. The range of results is given in parenthesis next to the mean
[b] Nine samples were analyzed
[c] Eleven samples were analyzed
[d] Six samples were analyzed
[e] Four samples were analyzed

6. Clearly some D-amino acids are present in this highly processed food material. Interestingly, recent studies have shown the presence of significant quantities of D-amino acids in certain foods consumed by man. This includes fermented products such as yogurt, some processed cheeses and other products (Bruckner and Hausch, 1990; Man and Bada, 1987). It is likely that the D-amino acid content of the rodent food used in this study (as well as other dry animal foods) resulted either from microbiological action on the protein, peptide and amino acid components at some point during the manufacturing processes and/or to physico-chemical effects of the manufacturing process (e.g., high temperatures and/or pH variations during consolidation, sterilization or drying, etc.). Another possible source of D-amino acids is from amino acid supplements that are sometimes added to animal feed. This is most often done for cattle feed and these supplemental amino acids generally are not the ones considered in this study.

Given the results in this and past studies some conclusions may be drawn concerning free D-amino acids in common laboratory rodents. First of all, there is no doubt that free D-amino acids are present in mammalian physiological fluids. The relative levels (of D to L-amino acids) is generally higher (frequently an order of magnitude or more) in the urine than in other physiological fluids such as plasma. It is likely that this is due to the fact that L-amino acids are more efficiently reabsorbed by the kidney tubules than are D-amino acids (Silbernagl, 1988; Young and Freedman, 1971). Under identically controlled conditions, different rodent strains excrete different relative amounts of D-amino acids. Also, each amino acid appears to have a characteristic D to L ratio. For example if a C57BL mouse excretes proline consisting of 44% of the D-enantiomer, the same relative excretion levels should not necessarily be expected for other amino acids (in fact they are less). A variety of factors can affect the relative amounts of D-amino acids in physiological fluids including diet, age, pregnancy, sickness, sex and antibiotics.

We feel that the origin of D-amino acids in physiological fluids is still open to considerable speculation. The results of this study seem to indicate that the diet is a major source of D-amino acids. Indeed it is possible that some of the factors that cause variations in D-amino acid levels (e.g., sickness and age) may be due at least in part to dietetic changes brought on by these various

conditions. Other of our results indicate that even if diet is the major source of D-amino acids in healthy rodents, it may not be the only source. Two other sources have been proposed previously. One is that the observed D-amino acids are produced by the gut bacteria and the other is that they are from the animal's own protein that has been degraded (possibly due to prior damage, age or other reasons) (Konno et al., 1990; Nagata and Akino, 1990). The facts that fasting lowers D-amino acids to constant levels, but does not eliminate them and administration of antibiotics causes erratic, unpredictable changes in relative D-amino acid levels could possibly be related to nondietary effects or sources (i.e., protein degradation or gut bacteria). It should be noted, however, that there is another possible explanation for antibiotic effects on amino acid excretion. If the antibiotic affects kidney function (even slightly or temporarily) in any way this could result in changes in the D/L excretion ratio. Hence the erratic changes noted upon antibiotic administration may have been the result of the kidney being somewhat affected by and than adjusting to an outside stimulus (i.e., the antibiotic or some effect caused by the antibiotic).

Another intriguing result of this work involves the relatively low level of D-amino acids found in newborn rodents and whether or not this is connected with the apparently elevated relative excretion of D-amino acids in pregnant mice. It is possible that the low levels found in newborn rodents simply reflects the fact that the food source for the fetus is from the mother's blood supply (already partially cleansed of D-amino acids via the kidney) and after it traverses an additional placental membrane (which may or may not further discriminate against D-amino acids). The reason pregnant rodents excrete higher relative levels of D-amino acids remains open to speculation. It is possible that this simply reflects the physiological changes that occur during pregnancy. It may also be possible that pregnant rodents have a greater need for L-amino acid "building-blocks" and therefore recovers them more efficiently. However, the simplest explanation may be that pregnant rodents eat more and therefore have greater amounts of D-amino acids to excrete. Whether or not excessive D-amino acid levels are detrimental to fetal development (and therefore are reduced *in vivo*) is unknown.

This work raises an interesting question as to whether a specific D,L-amino acid ratio can be used to test certain aspects of kidney function. In an analogous procedure to the glucose tolerance test for diabetes, an excess quantity of a D-amino acid could be ingested and subsequently the urine or blood could be tested for appearance, clearance, etc. With the current procedures, "normal" metabolic and catabolic patterns should be easy to determine. Subsequently, any aberrations could be spotted in a straghtforward and reproducible manner.

Although we feel a good deal had been learned about D-amino acids in mammals, a number of intriguing questions have arisen as well. Clearly these will benefit from further study.

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