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D-Amino acids in chronic renal failure and the effects of dialysis and urinary losses

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Summary. Total D-amino acids were measured in plasma for 20 non-dialysed patients (creatinine clearance < 12 ml/minute), 20 on CAPD, 20 on haemodialysis and 20 normals. Plasma D-tyrosine and D-phenylalanine were measured in 8 of each group by HPLC. Total D-amino acids, D-tyrosine and D-phenylalanine were significantly greater for patients than normals. D-amino acids and D-tyrosine correlated with creatinine and were decreased during HD. During dialysis, the mean losses for D-tyrosine and D-phenylalanine were similar, about 0.2 mg/CAPD exchange and 3 mg/4 hour haemodialysis (i.e. 2% of the total amino acid, as in plasma). Clearance was unaffected by stereochemical configuration. Urinary losses/24 hour in the non-dialysed patients were 0.35 mg D-tyrosine and 0.25 mg D-phenylalanine. Clearance for D-phenylalanine was greater than for the L-enantiomer. Increases in D-amino acids in renal failure are probably due to depletion of D-amino acid oxidase, but may be enhanced by a D-amino acid rich diet, peptide antibiotics and D-amino acid oxidase inhibiting drugs and metabolites. Possible toxic effects need further investigation.

Keywords: Amino acids – Chronic renal failure – Plasma – Urine – D-amino acids – CAPD – D-Tyrosine – D-Phenylalanine – Haemodialysis

Introduction

All amino acids, except for glycine, occur as optically active isomers and rotate the plane of polarised light to the left (laevorotatory) or to the right (dextrarotatory). Amino acids usually used in protein synthesis are of the L-configuration whereas D-amino acids rarely occur in proteins. During the past thirty years, D-amino acids have been detected in micro-organisms (Meister, 1965), some insects (Corrigan and Srinivasan, 1966), marine invertebrates (Preston, 1987), higher plants (Robinson, 1976), various mammals including rodents and guinea pigs (Hoeprich, 1965) and also in humans (Nagata et al., 1987). D-aspartate accumulates in stable proteins such as tooth enamel (Helfman and Bada, 1975)

the eye lens (Masters et al., 1977) and white cerebral tissue (Man et al., 1983) due to in vivo racemisation. Despite the presence of D-amino acid oxidase in the liver, kidney, brain and plasma (Barker and Hopkins, 1977) most free D-amino acids are present in plasma (Nagata et al., 1992, Brückner and Hausch, 1993) and they are excreted in the urine (Armstrong et al., 1991; Ketting et al., 1991). In humans, only the D-forms of methionine and phenylalanine can be converted, to some extent, to their L-forms.

The presence of D-amino acids in plasma was reported by Negata et al., (1987) who found that the concentrations in patients with renal disease were increased in proportion with plasma creatinine and β2-microglobulin. In a later study 12 neutral amino acids were surveyed for the presence of D-enantiomers using HPLC, and D-serine, D-alanine and D-proline were higher than for controls. A simultaneous study by Brückner and Hausch (1993) confirmed these observations in patients with renal disease including those on haemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD). D-glutamic acid, D-aspartic acid and D-leucine were also measured in several patients.

The aim of this study was to investigate the concentrations of total D-amino acids, D-tyrosine and D-phenylalanine in comparable groups of non-dialysed patients with creatinine clearances less than 12 ml/min, HD and CAPD patients and individuals with normal renal function. Plasma, dialysates and urine were analysed to establish the effective clearance of D-amino acids by dialysis and urinary excretion.

Material and methods

Patients

80 individuals were investigated: 20 individuals with normal renal function, 20 patients with creatinine clearances less than 12 ml/min (non-dialysed), 20 on CAPD and 20 on HD with equal numbers of men and women in each group except for the non-dialysed group which had 13 males and 7 females. The mean ages for each group were: normals 49.6 $\pm$ 10.8 years, non-dialysed 57.8 $\pm$ 15.9, CAPD 50.6 $\pm$ 16.6 years, HD 60.7 $\pm$ 10.8 years (v normals $p < 0.002$). The dialysis patients had been maintained on their treatment for at least 3 months and had remained free from peritonitis or other infections for at least one month before this study.

Methods

Non-fasting blood was collected in ethylene diamine acetic acid (edta) tubes over ice and the plasma was separated immediately and stored at -80°C. Blood samples from CAPD patients were collected before the afternoon exchange at about 1400 hrs, together with aliquots of dialysate for 8 patients from the morning exchange (2 litres), also collected on ice and centrifuged. Blood samples from HD patients were collected before and after dialysis, about 1000 and 1400 hrs, together with aliquots from the total spent dialysis fluid of about 150 litres. 24 hr urines were collected from 8 of the non-dialysed patients. All dialysates and urine were also stored at -80°C.

Total plasma D-amino acids were measured by the D-amino acid oxidase method (Nagata et al., 1987) and plasma creatinine using a Clinical System 700 (Beckman Instruments, California).

Plasma tyrosine, phenylalanine and the D- and L-enantiomers were measured in 8 individuals from each group (4 males and 4 females) using a modification of the method

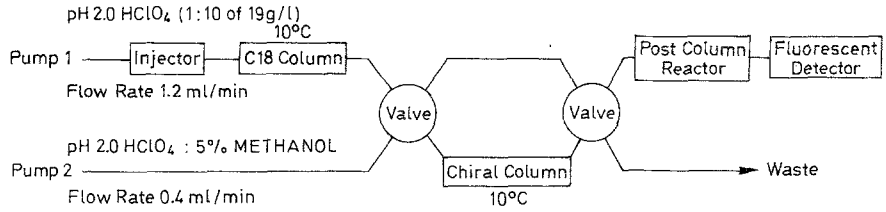

Fig. 1. Scheme showing a coupled column system for measuring plasma tyrosine, phenylalanine and the D- and L-enantiomers (modification of the method of Armstrong, 1991)

described by Armstrong et al. (1991). The Waters HPLC system (Millipore Corporation, Milford, M.A.) consisted of Model 510 pumps, an Automated Gradient Controller, a Wisp 710B, a 740 Data module, a 470 Fluorescent Detector and two 4-port valves (Fig. 1). Plasma, dialysates and urines were ultrafiltered using Ultrafree PG filters with a 10,000 nominal molecular weight limit (Millipore Corporation, Milford, M.A.). 2 μl of ultrafiltered plasma or standard (0.125 μmol/ml DL-phenylalanine and 0.125 μmol/ml DL-tyrosine) or 2–20 μl of dialysate or urine was injected. Amino acids were separated using 19 g/l of 60% perchloric acid diluted 1:10 to pH 2.0 and run at a flow rate of 1.2 ml/min on a 10 cm C18 column (50DS from Technicol, Stockport). The column was cooled to 10°C to delay the elution of tyrosine and phenylalanine as discrete peaks (Fig. 2). These were measured after post-column derivatisation using ortho-phthalaldehyde reagent on the fluorescent detector. A second

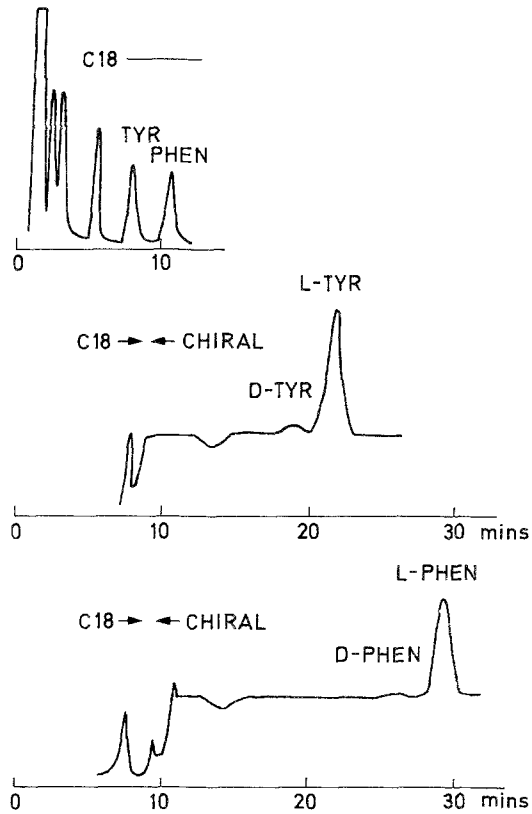

Fig. 2. A C18 chromatogram to isolate tyrosine and phenylalanine and subsequent chiral separation of the D- and L-enantiomers

Table 1. Mean (±SD) plasma concentrations of creatinine, total D-amino acids and values for tyrosine and phenylalanine (i.e. total (D + L), D/L ratio (%), D-enantiomer) in normal individuals, non-dialysed, CAPD and HD patients

N = 20	Normals	Non-dialysed	CAPD	HD	
				Pre	Post
Creatinine	87.5 ± 25.0	604 ± 181***	858 ± 359***	964 ± 335***	437 ± 196###
D-amino acids μmol/l	9.2 ± 8.2	16.1 ± 5.9***	18.3 ± 5.8***	25.2 ± 159***	16.9 ± 8.1###
n = 8					
Tyrosine:					
(D + L) μmol/l	80 ± 13.2	60 ± 18.6*	61 ± 16.1**	55 ± 16.8	45 ± 8.0
(D/L) %	1.2 ± 0.3	5.3 ± 2.6***	2.6 ± 1.2***	4.5 ± 2.0***	3.0 ± 2.0
(D) μmol/l	0.9 ± 0.16	3.3 ± 2.3**	1.5 ± 0.7**	2.3 ± 1.2**	1.2 ± 0.7#
Phenylalanine:					
(D + L) μmol/l	56 ± 10.2	68 ± 13.1*	75 ± 13.8**	59 ± 13.3	57 ± 7.9
(D/L) %	1.0 ± 0.4	1.5 ± 0.7*	1.7 ± 1.2	3.1 ± 2.2**	2.9 ± 1.3
(D) μmol/l	0.5 ± 0.2	1.0 ± 0.4**	1.1 ± 0.6*	1.8 ± 1.7*	1.6 ± 0.9

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.002$ compared with normal
\# $p < 0.05$, ## $p < 0.002$, ### $p < 0.002$ compared with pre-haemodialysis

pump was run simultaneously with perchloric acid (pH 2.0) containing 5% methanol at a flow rate of 0.4 ml/minute through a Crownpack CR (+) column (JT Baker, Phillipsburg, NJ) also cooled to 10°C. During separate runs tyrosine and phenylalanine were transferred from the C18 to the Chiral column, by means of opening the first valve for 30 seconds, to separate the isomers. Simultaneous adjustment of the second valve allowed incorporation of post-column derivatisation and fluorescent detection whilst the second pump was running. Each run lasted about 40 minutes (Fig. 2). The preparation and analysis of samples was designed to minimise racemisation.

Data are presented as means ± SD. Differences between the groups were compared with normals using the two-tailed Mann-Whitney U-test for unpaired data. Paired data was compared using the Wilcoxon's rank sum test. Correlation coefficients were determined by the Spearman Rank test.

Results

The mean (±SD) plasma concentrations of creatinine, D-amino acids, D-tyrosine and D-phenylalanine for individuals with normal renal function and non-dialysed, CAPD and HD patients are shown in Table 1 and Figs. 3 and 4.

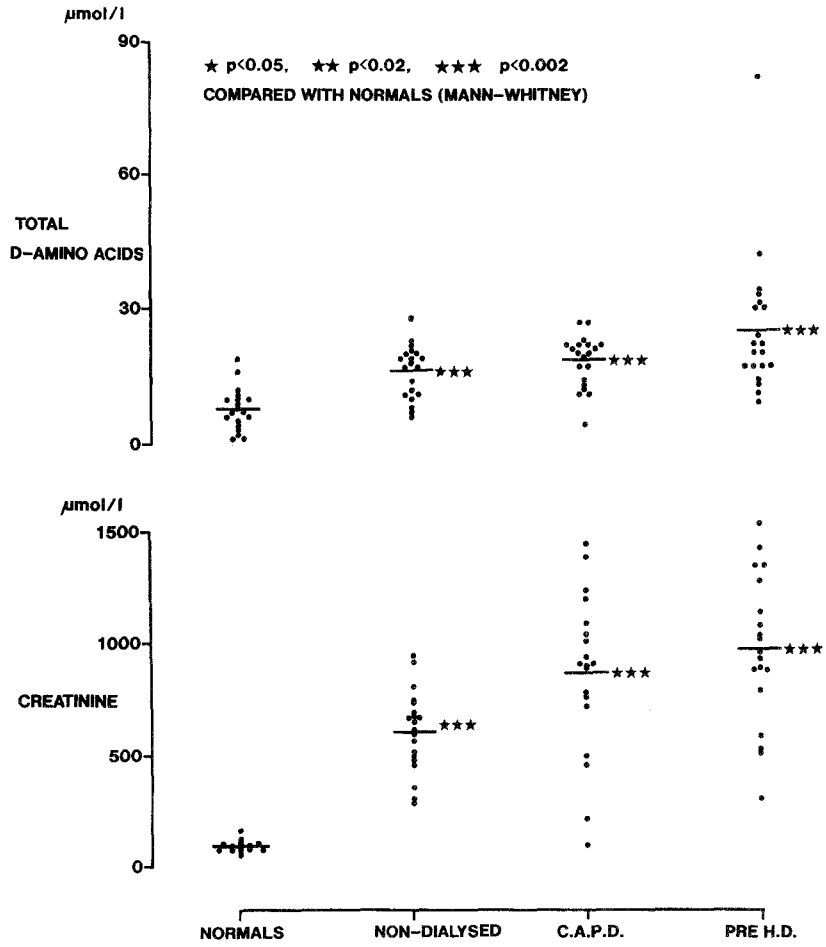

Fig. 3. Plasma D-amino acids and creatinine in normals and non-dialysed, CAPD and HD patients. Means and significant differences from normal are shown

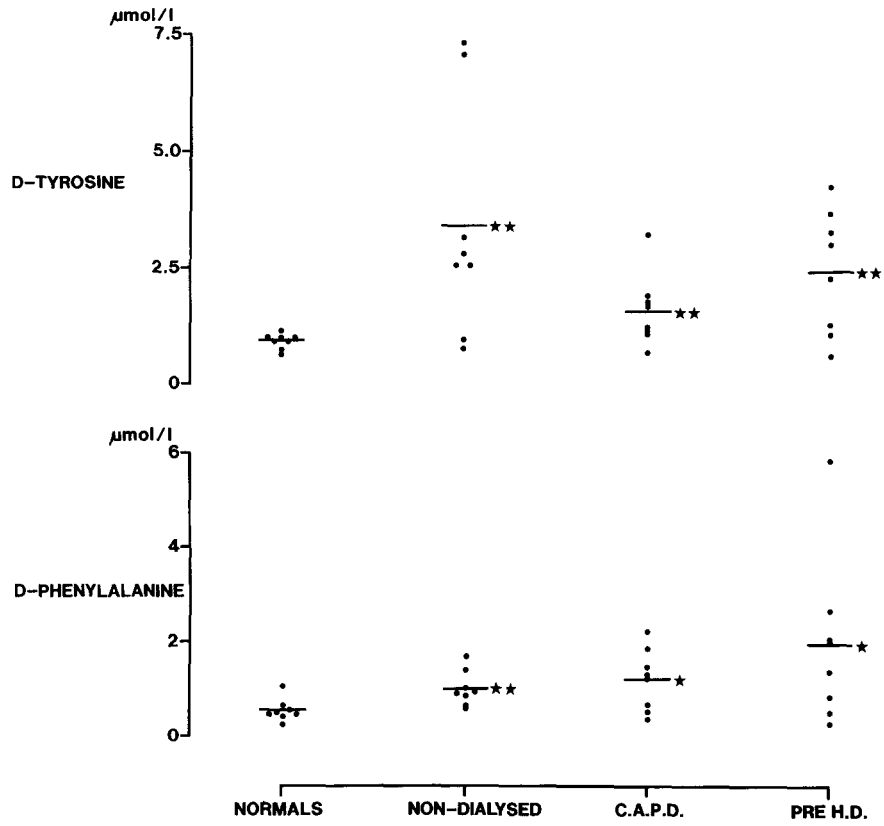

Fig. 4. Plasma D-tyrosine and D-phenylalanine in normals and non-dialysed, CAPD and HD patients. Means and significant differences from normal are shown

The mean values for total D-amino acids, D-tyrosine and D-phenylalanine in non-dialysed CAPD and HD patients were significantly greater than for individuals with normal renal function. The increases for plasma D-tyrosine were greater than for D-phenylalanine in the non-dialysed patients.

Patients on HD were significantly older than the normals and this could contribute slightly to the higher concentrations of D-amino acids in this group. D-amino acids correlated with age for all the groups combined ($R = 0.29$, $p < 0.01$) but no correlations were observed for individual groups. D-tyrosine and D-phenylalanine were unrelated to age. Plasma creatinine correlated with D-amino acids in all the groups combined ($R = 0.48$, $p > 0.001$) and also with D-tyrosine ($R = 0.51$, $p < 0.02$). Plasma creatinine also correlated with D-amino acids for the non-dialysed group ($R = 0.51$, $p > 0.02$) but not for the normal, CAPD or HD groups.

The mean ($\pm$SD) plasma concentrations of D-amino acids, D-tyrosine and D-phenylalanine, before and after HD are shown in Table 1 and Fig. 5. Plasma D-amino acids and D-tyrosine were decreased but the change in D-phenylalanine was not significant.

The mean losses ($\pm$SD) and clearances for the enantiomers of tyrosine and phenylalanine into dialysate during CAPD and HD are shown in Table 2. During CAPD the loss per exchange of both D-enantiomers was 0.2 mg and

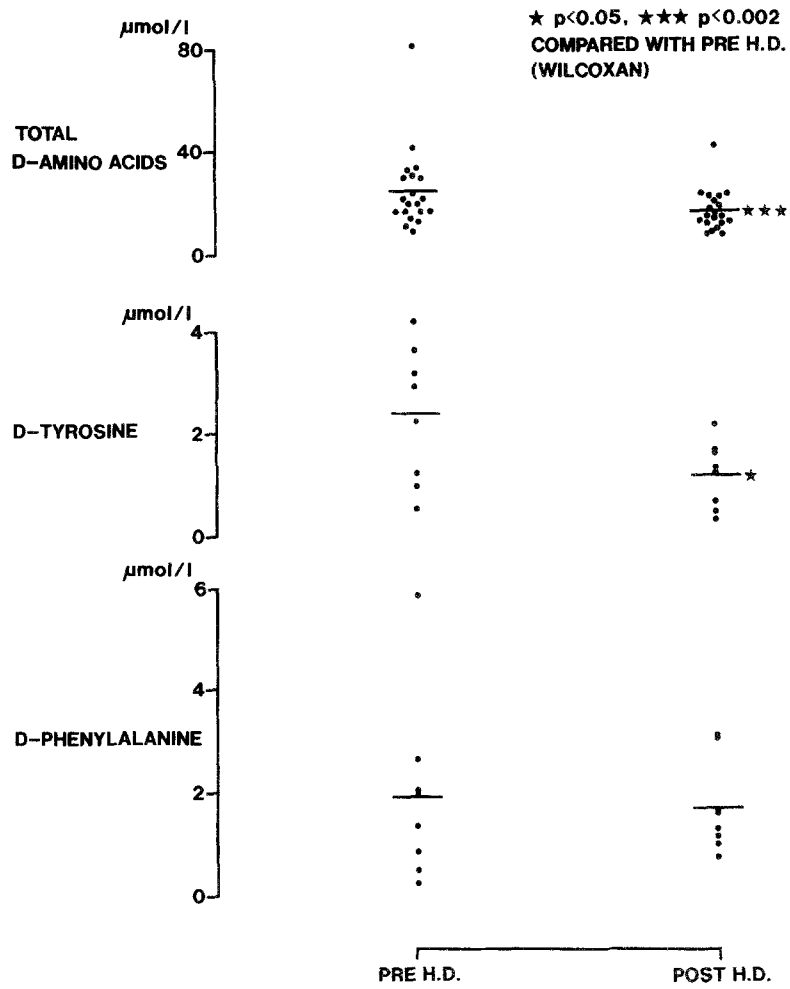

Fig. 5. Plasma D-amino acids, D-tyrosine and D-phenylalanine before and after haemodialysis

Table 2. Mean (±SD) losses and clearances for the enantiomers of tyrosine and phenylalanine into dialysate during CAPD and HD

	CAPD Dialysate		HD Dialysate	
	Loss/exchange mg	Clearance ml/min	Loss/dialysis mg	Clearance ml/min
L-Tyrosine	8.9 ± 6.0	4.1 ± 2.4	144 ± 83	86 ± 26
D-Tyrosine	0.18 ± 0.11 (2.0%)	3.4 ± 1.7	3.5 ± 1.4 (2.4%)	81 ± 28
L-Phenylalanine	10.1 ± 4.8	3.7 ± 1.7	178 ± 56	83 ± 21
D-Phenylalanine	0.18 ± 0.08 (1.8%)	4.0 ± 1.8	2.9 ± 1.1 (1.6%)	84 ± 24

during HD the loss per dialysis was 3.6 mg of D-tyrosine and 2.9 mg D-phenylalanine. These loses were about 2% of the total for each amino acid and similar to the proportions present in plasma. Thus there were no significant differences in the clearance of the enantiomers during either CAPD or HD.

Table 3. Mean (± SD) losses and clearances for the enantiomers of tyrosine and phenylalanine in 24 hour urines in non-dialysed patients

	Non-dialysed patients (urine)	
	Loss/24 hours mg	Clearance ml/min
L-Tyrosine	11.0 ± 7.5	0.75 ± 0.44
D-Tyrosine	0.39 ± 0.26 (3.5%)	0.65 ± 0.50
L-Phenylalanine	9.7 ± 4.8	0.61 ± 0.30
D-Phenylalanine	0.28 ± 0.12 (2.9%)	1.34* ± 1.10

* $p < 0.05$ compared with L-phenylalanine

The mean losses (± SD) and clearances for the enantiomers of tyrosine and phenylalanine in 24 hr urines for the non-dialysed patients are shown in Fig. 3. The loss per 24 hrs of D-tyrosine was 0.35 mg and D-phenylalanine was 0.25 mg. The urinary clearance of D-phenylalanine was significantly greater than for the L-enantiomer ($p < 0.05$).

Discussion

The presence of D-amino acids in the plasma of individuals with normal renal function and the increases in patients with chronic renal failure, in proportion with plasma creatinine, are in agreement with the observations of Negata et al. (1987). Our study also shows that small but measurable amounts of D-tyrosine and D-phenylalanine are present in the plasma of individuals with normal renal function and that they are increased in chronic renal failure in non-dialysed and dialysed patients. Such increases occur despite some clearance in urine and dialysis fluid. Consequently, several questions arise concerning the origin of these D-amino acids, the role of the kidney in their removal and their possible toxicity.

Available evidence suggests that D-amino acids in humans may originate in three ways: endogenously by catabolism of long life proteins in which L-amino acids have been racemised to the D-form; release from the gastrointestinal tract following racemisation by bacteria; and from dietary sources.

The catabolism of long life proteins that are in tooth enamel, human lenses and human brain (Negata et al., 1992) and other proteins, as yet unidentified, may be a source of D-amino acids. Increased catabolism does occur in severely uraemic and malnourished patients and may contribute to increased concentrations. However, the number of amino acids and the quantity released from proteins incorporated in some of these tissues would be small as they require acid hydrolysis (Brückner and Hausch, 1993).

Gastrointestinal bacteria are a source of D-amino acids as they are present both in cell walls and cytoplasm, whilst racemases convert most L-amino acids into their D-enantiomers (Meister, 1965). An intestinal origin for D-alanine is supported by Heoprich (1965) who was unable to find any in the sera of germ free guinea pigs and mice. Furthermore, an increased urinary excretion of

D-alanine occurs in the short bowel syndrome (Ketting et al., 1991), and 15N-labelled intestinal microbial amino acids appear in the venous blood of pigs (Niiyama et al., 1979). However, in contrast to these observations Nagata and Akino (1990) did find D-amino acids widely distributed in germ free mice suggesting additional endogenous sources. The bacterial infection of tissues could also be a source of D-amino acids (Ueda et al., 1989) and increase concentrations in chronic renal failure.

Dietary intake may be a more important source of D-amino acids than previously thought. Various processes are used in the food industry to improve taste, texture and shelf life, and these include heat and alkali treatments which favour the formation of D-amino acids. A wide range have been found in such foods as soy and corn protein products, casein, cooked chicken, toast, and the artificial sweetener aspartame (Man and Buda, 1987). Fermented foods such as yoghurt, cheese, wine and beer are also important sources (Brückner et al., 1992). High plasma levels in chronic renal failure are not attributable to high dietary intake in nondialysed patients because they are maintained on low protein diets. However, high concentration may occur in individuals who consume a high proportion of foods rich in D-amino acids. The use of peptide antibiotics containing D-amino acids or metabolites or drugs that inhibit D-amino acid oxidase (Hamilton and Buckthal, 1982) could cause an increase in plasma concentration. These inhibitors include a wide range of compounds such as diuretics e.g. furosemide and mersalyl; anti-inflammatory agents, e.g. salicylate and indomethacin; hypoglycaemic, hypocalcaemic and hypolipidaemic compounds and nucleotides.

Increased concentrations of D-amino acids in chronic renal failure can clearly occur even when low protein diets are given. The filtration and excretion of amino acids is reduced in patients with a low glomerular filtration rate. Furthermore, a preferential retention of D-amino acids occurs due to the loss of proximal tubular cells containing D-amino acid oxidase. The greater increase for D-tyrosine than for D-phenylalanine in the non-dialysed patients can be attributed to two factors. Firstly, D-phenylalanine can be converted to the L-form whereas D-tyrosine cannot (Lehmann et al., 1983) and secondly, our results show that D-phenylalanine has a higher urinary clearance.

Some adverse effects of diets containing D-amino acids in small animals with normal renal function have been reported although blood concentrations were not measured. Growth studies on mice fed synthetic amino acid diets containing D-tyrosine severely depressed weight gain and eventually led to their deaths (Friedman and Gumbmann, 1984). The authors considered possible ways in which tyrosine could act as an antimetabolite and one was interference with the hydroxylation of L-phenylalanine to L-tyrosine. Previous studies have shown that such an inhibitor exists in chronic renal failure and could contribute to a low tyrosine concentration (Young and Parsons, 1972).

Most D-amino acids, particularly D-glutamic acid act as immunosuppressive agents in mice (Inoue et al., 1981). However, measurements of spleen index and LD50 values in this study showed no toxicity. D-serine and D-proline, both of which are present in chronic renal failure (Negata et al., 1987; Brückner and Hausch, 1993) may be exceptions to this. D-serine causes necrosis of the straight

segment of the proximal tubule in the rat kidney accompanied by diuresis, proteinuria and aminoaciduria (Artom et al., 1945; Ganote et al., 1974). D-proline causes severe renal and hepatic damage in rats and can be lethal in chicks (Kampel et al., 1990). Clearly any attempts to relate any of these effects to the concentrations found in patients with chronic renal failure are speculative and further investigations are needed.

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