Diabetologia (1993) 36: 797–801

**Diabetologia**



# D-Lysine reduces the non-enzymatic glycation of proteins in experimental diabetes mellitus in rats

**M. Sensi[1], M. G. De Rossi[1], F. S. Celi[1], A. Cristina[1], C. Rosati[1], D. Perrett[2], D. Andreani[1], U. Di Mario[3]**

[1] Cattedra di Medicina Costituzionale ed Endocrinologia, Università degli Studi di Roma "La Sapienza", Rome, Italy
[2] Medical Professorial Unit, St. Bartholomew's Hospital Medical School, London, UK
[3] Dipartimento di Medicina Sperimentale e Clinica, Università di Reggio Calabria, Catanzaro, Italy

**Summary.** D-Lysine, the non-physiological isomer of L-lysine, can competitively reduce protein non-enzymatic glycation in vitro. To study the effect of D-lysine in vivo, 6–8-week old Sprague-Dawley rats with streptozotocin-induced diabetes mellitus were treated from diagnosis for 45 days with two daily subcutaneous injections of D-lysine ($0.5\ g \cdot ml^{-1} \cdot day^{-1}$). Another group of diabetic rats was only injected with equal volumes of physiological saline (0.9% NaCl). Glycated haemoglobin was measured by ion exchange chromatography, and glycated serum and lens proteins by boronate affinity gel chromatography. Serum and urinary creatinine concentrations were evaluated by the alkaline-picrate reaction. Urinary lysine concentrations at mid- and end-study were evaluated by cation exchange chromatography. Blood glucose concentrations, serum creatinine levels and creatinine clearances, measured at the end of the study, were similar in both diabetic groups ($> 22.0$ mmol/l, $\leq 106$ μmol/l and $\approx 0.02$ ml/s, respectively). Urinary lysine concentration in D-lysine-treated diabetic animals was more than 50-fold higher than in placebo-treated diabetic rats. In D-lysine-treated vs placebo-treated diabetic animals, a statistically significant reduction was found in the levels of glycated haemoglobin (stable $HbA_1$; mean ± SD = 3.00 ± 0.74% vs 4.02 ± 0.46%, $p < 0.05$; labile $HbA_1$ = 3.92 ± 0.89% vs 5.84 ± 0.61%, $p < 0.005$), glycated serum proteins (1.40 ± 0.47% vs 2.52 ± 1.15%, $p < 0.05$) and glycated lens proteins (4.90 ± 0.96% vs 5.98 ± 0.65%, $p < 0.05$). Thus, D-lysine (i) is not nephrotoxic and (ii) causes a significant reduction of the early glycation products at the protein level. Therefore, the D-amino acid could be useful in attempting to control damaging phenomena associated with or due to an enhanced protein non-enzymatic glycation.

**Key words:** D-Lysine, Maillard reaction, non-enzymatic glycation, proteins, diabetes mellitus, streptozotocin rat model.

Structural and functional characteristics of proteins can be altered by a series of interrelated chemical processes collectively known as "the Maillard reaction" [1]. The initial step of this reaction consists of a non-enzymatic condensation between ambient glucose – in the open (aldose) form – and free protein terminal or side chain (mainly those of L-lysine) amino groups. It is now believed that the final adducts of the Maillard reaction, namely the late Amadori products or advanced glycation end-products (AGE), may be involved in physiological aging [2–4] and, in the case of diabetes mellitus with its associated hyperglycaemia, in the pathogenetic sequelae leading to the late complications of this disease [5, 6]. Thus, pharmacochemical interventions, intended to modify the extent of the initial non-enzymatic glycation reaction, are being actively investigated. Some of the products which have been studied in vitro and, in some cases, in vivo, are acetyl-salicylic acid [7, 8], vitamin C [9, 10] and Bendazac [11], although the most successful thus far is aminoguanidine [12–15]. It is believed that these compounds are capable of reducing the non-enzymatic glycation process either by interfering with glucose attachment to the free reaction sites on protein or by blocking further steps of the reaction. The main problem arising from this type of competitive effect is that other "foreign" chemical groups, instead of glucose, may become attached to proteins. This, in the long run, could hypothetically lead to functional and structural protein alterations, possibly just as damaging as those caused by glucose.

We have approached the problem from a different angle: to try to reduce the extent of non-enzymatic glycation by actively subtracting some of the reactive glucose (the open aldose form with the free carbonyl group, which makes up only a very small proportion of circulating sugar molecules, since, at any time, most of the glucose is in the form of the closed pyranose ring [16]) from the protein reacting sites. For this purpose the dextro-isomer of L-lysine, D-lysine, has been chosen, on the principle that it pos-

**Table 1.** Metabolic and clinical chemical parameters measured in diabetic animals treated with D-lysine (Group A), diabetic animals treated with placebo (Group B) and untreated control animals (Group C)

| | Group A | Group B | Group C |
|---|---|---|---|
| Blood glucose (mmol/l) | 25.1 ± 3.8[a] | 22.8 ± 4.1[a] | 5.5 ± 0.5 |
| 24-h urine volume (ml) | 39 ± 13 | 35 ± 9 | 26 ± 7 |
| Body weight increase (%) | 91 ± 15[a] | 95 ± 9[a] | 151 ± 16 |
| Serum creatinine (μmol/l) | 97 ± 9 | 97 ± 26 | 106 ± 18 |
| Creatinine clearance (ml/s) | 0.02 ± 0.003 | 0.02 ± 0.008 | 0.02 ± 0.01 |
| 24 h-urine lysine concentration (mmol/l) | | | |
| Mid-experiment | 20.1 ± 2.2 | 0.2 ± 0.1 | (not measured) |
| End-experiment | 15.9 ± 1.5 | 0.3 ± 0.3 | 0.2 ± 0.1 |

[a] $p < 0.0001$ vs control group C (unpaired two-tailed Student's $t$-test)

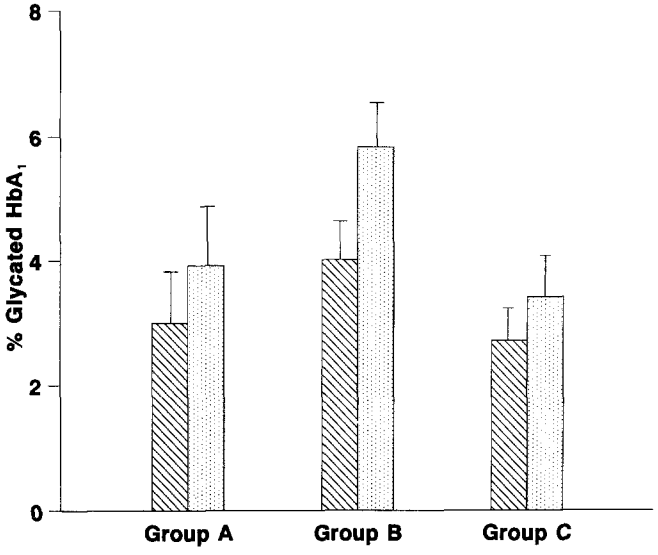


**Fig. 1.** Effect of D-lysine on non-enzymatic glycation of $HbA_1$. The percent glycation of both stable (▨) and labile (▩) forms of $HbA_1$ in D-lysine-treated diabetic animals (Group A) is significantly reduced (see below) by comparison with placebo-treated (Group B) diabetic animals and is not different from that measured in normal animals (Group C). Group statistical analysis. One-way ANOVA: (stable $HbA_1$) $F$ ratio = 7.064, $df = 14$, $p < 0.01$; (labile $HbA_1$) $F$ ratio = 15.524, $df = 14$, $p < 0.0005$. Unpaired one-tailed $t$-test: (stable $HbA_1$) Group A ($n = 5$) vs Group B ($n = 5$), $df = 8$, $p < 0.05$; Group A vs Group C ($n = 5$), NS; Group B vs Group C, $p < 0.002$. (labile $HbA_1$) Group A vs Group B, $p < 0.005$; Group A vs Group C, NS; Group B vs Group C, $p < 0.0002$

sesses the same chemical characteristics as the levo-form, but is physiologically inactive and is excreted mainly by glomerular filtration [17, 18]. In two in vitro studies we have shown that the inhibitory action of D-lysine on non-enzymatic glycation is specific and amino acid concentration-dependent [19] and is effective in reducing AGE levels [20]. In the present study we have investigated the inhibitory action of D-lysine on the formation of the early products of the Maillard reaction in vivo in an animal model of diabetes.

## Materials and methods

### *Animals*

A total of 10 male Sprague-Dawley rats, bred in the animal care facility of the Università Cattolica del Sacro Cuore, Rome, Italy, weighing between 250–300 g (age: 6–8 weeks) were made diabetic by a single i.p. injection of streptozotocin, at a dose of 60 mg/kg body weight. Five rats of the same sex, weight and age were used as normal controls.

### *Experimental protocol*

Treatment with D-lysine was begun immediately at onset of hyperglycaemia (5–6 days after streptozotocin administration). Diabetic animals were randomly divided into two groups. Group A received two daily s.c. injections (mid-morning and mid-afternoon) of D-lysine monohydrochloride (Sigma Chimica, Milan, Italy) at a concentration of 0.250 g in 0.5 ml sterile distilled water, brought to pH 7.3 with NaOH (1.0 mol/l). Group B was similarly injected with an equal volume of physiological saline (0.9% NaCl, pH 7.3). Group C, consisting of normal rats, received no treatment and was used as baseline. Five bilateral injection sites were selected on the animal backs and changed daily on a rotation system, so that each site was reinjected at 5-day intervals, to cause as little dermal tissue irritation as possible. Treatment with D-lysine and placebo was continued for 45 days. At completion of the study, all the animals were killed under ether anaesthesia and two blood samples (one with and the other without anti-coagulant) collected to obtain haemoglobin and serum proteins, respectively. Lenses, as source of proteins with sizeable amounts of the early products of the Maillard reaction (ketoamines or early Amadori products), were also removed and stored in liquid $N_2$ until use.

### *Metabolic parameter determination*

Initial and final blood glucose levels were determined by Haemoglucotest 20–800 R strips (Boehringer Mannheim Italia S.p.A., Milan, Italy). Animal body weights were checked and recorded throughout the study at weekly intervals. At experiment mid-point (23 days) and at its completion, all animals were placed in metabolic cages for 24-h urine collection.

### *Clinical chemical parameter determination*

*Urinary lysine concentration:* the amino acid concentration in the 24-h urine samples was determined by cation exchange chromatography, with post-column fluorescence detection using o-phthalaldehyde/N-acetyl-cysteine. The method does not distinguish between D- and L-amino acids [21].

*Creatinine:* the plasma and urinary levels of creatinine were measured by the alkaline-picrate reaction (Creatinine SERA-PAK; Miles Italiana S.p.A., Divisione Ames, Milan, Italy)

### *Quantitative evaluation of glycated proteins*

### *Preparation of protein samples*

*Haemoglobin and serum proteins:* heparinised blood samples were centrifuged at 400 × g for 10 min. The plasma and the buffy coat were discarded and 0.1 ml of packed erythrocytes removed and washed twice with 1.0 ml physiological saline. Following the removal of the last wash supernatant, 0.05 ml of washed packed erythrocytes

were lysed with 1.0 ml ice-cold distilled water. After centrifugation at 4 °C for 10 min at 400 × g, the clear erythrocyte lysate containing haemoglobin was removed and stored at −80 °C for further use. Non-heparinised, clotted blood samples were centrifuged for 10 min at 400 × g and the serum removed. Excess glucose in the serum samples was removed by 24-h dialysis at 4 °C against 1 litre physiological saline. The dialysed serum samples were then stored at −80 °C for further use.

*Lens water-soluble proteins:* lenses were homogenised with a hand-held glass homogeniser in 2.0 ml de-aereated phosphate buffered saline (PBS 0.15 mol/l: NaCl, 8.00 $g \cdot l^{-1}$; KCl, 0.20 $g \cdot l^{-1}$; $Na_2H\,PO_4$, 1.15 $g \cdot l^{-1}$; $KH_2PO_4$, 0.20 $g \cdot l^{-1}$), pH 7.3. The lens homogenates were dialysed for 24 h at 4 °C against 1 litre PBS and centrifuged at 4 °C for 2 h at 10,000 × g. The supernatant, containing water-soluble lens proteins, was removed and stored at 4 °C before use.

### *Determination of glycated protein levels*

Percent glycated haemoglobin was measured by ion exchange chromatography (A. Menarini, Divisione Diagnostici, Florence, Italy), a method which discriminates between the stable (s) and the labile (l) forms of $HbA_1$. Serum and lens glycated proteins were separated from their non-glycated forms by means, of boronate affinity gel chromatography using the Glyco. Gel B test kit (Pierce Chemical Company, Rockford, Ill., USA) [22]. The protein concentration in each serum and lens fraction was spectrophotometrically determined (absorbance readings set at 280 nm), thus allowing calculation of the percent glycation.

### *Statistical analysis*

The data, expressed as mean ± SD, were analysed by means of the one-way ANOVA for differences among treatment groups. Where a statistical significance was found ($p \leq 0.05$), the differences between treatment groups were analysed with the unpaired, one-tailed (unless otherwise stated) Student's *t*-test.

## Results

The metabolic and the clinical chemical parameters measured in the three groups of animals are shown in Table 1. Final blood glucose levels were significantly higher and the percent increase in body weight significantly lower in both diabetic groups A and B as compared to the normal control group C. Urine outputs (24-h) were also higher in diabetic animals, but did not reach statistical significance. No differences were observed between diabetic groups A and B. Serum creatinine levels and creatinine clearances were not different among the three groups. Urinary lysine concentrations at mid-point or end-point of the experiment were significantly increased in the diabetic group A as compared to diabetic group B and normal control group C.

The percentage of glycated haemoglobin ($HbA_1$ s and $HbA_1$ l), serum and lens proteins measured in the three animal groups are shown in Figures 1, 2 and 3, respectively. In each case, glycated protein levels are significantly lower in the diabetic group A (D-lysine-treated) as compared to the diabetic group B (placebo-treated). Moreover, no difference is observed when diabetic group A values are compared to the normal baseline values of group C.

## Discussion

D-Lysine treatment in diabetic animals has positively effected the reduction of non-enzymatic glycation of the selected protein types. This is evident even if the design of this in vivo study only allowed the determination of the early Amadori by-products of the Maillard reaction.

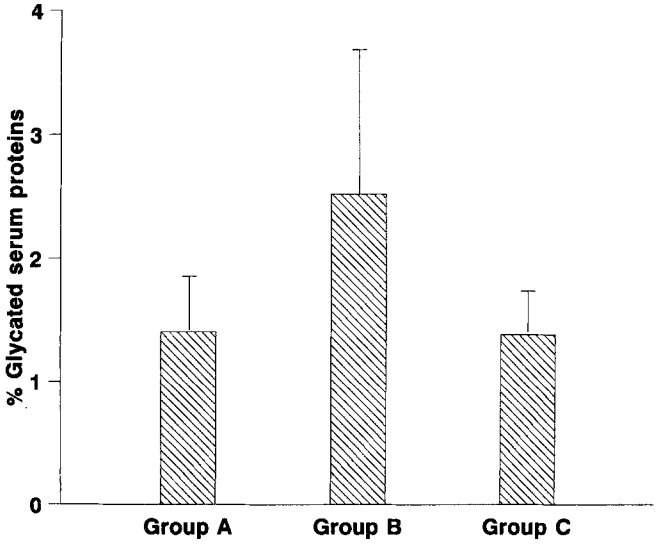


**Fig. 2.** Effect of D-lysine on non-enzymatic glycation of serum proteins. The percent serum protein glycation in D-lysine-treated diabetic animals (Group A) is significantly reduced (see below) by comparison with placebo-treated (Group B) diabetic animals. No difference is found when group A and group C (control animals) values are compared. Group statistical analysis: One-way ANOVA: $F$ ratio = 3.781, $df = 14$, $p = 0.05$. Unpaired one-tailed t-test: Group A ($n = 5$) vs Group B ($n = 5$), $df = 8$, $p < 0.05$; Group A vs Group C ($n = 5$), NS; Group B vs Group C, $p < 0.05$

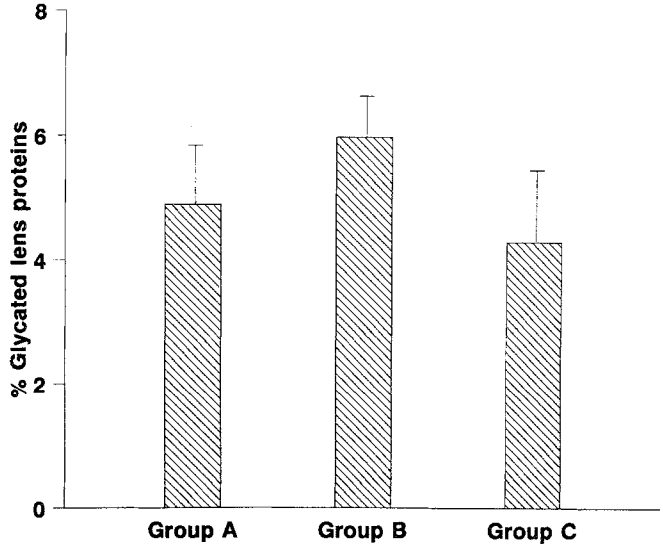


**Fig. 3.** Effect of D-lysine on non-enzymatic glycation of lens proteins. The percent lens protein glycation in D-lysine-treated diabetic animals (Group A) is significantly reduced (see below) by comparison with the placebo-treated (Group B) diabetic animals. No difference is found when group A and group C (control animals) are compared. Group statistical analysis: One-way ANOVA: $F$ ratio = 4.038, $df = 14$, $p < 0.05$. Unpaired one-tailed t-test: Group A ($n = 5$) vs Group B ($n = 5$), $df = 8$, $p < 0.05$; Group A vs Group C ($n = 5$), NS; Group B vs Group C, $p < 0.01$

An important condition for the occurrence of an inhibitory action on non-enzymatic glycation is, in the case of D-lysine, a high blood level of the amino acid, possibly throughout the day. Some studies on amino acid load have clearly demonstrated a toxic effect on kidney function [23, 24]. These studies were however performed using a fairly rapid i.v. administration of the amino acids [24]. Although the total daily amount of D-lysine injected in our experiment was extremely large (0.5 g), since we wanted (a) to achieve a circulating level high enough to produce an evident inhibitory effect and (b) to gain information about local tissue damage, the steady reabsorption into the blood stream from the s.c. injection sites is probably slow and would not lead to an abrupt renal load. Moreover, it has been shown that the nephrotoxicity of some D-amino acids, such as D-serine or D-proline, is directly correlated to the proximity of the amino group to the side chain group [25]. However, lengthening the backbone carbon chain has been shown to progressively reduce nephrotoxicity [25]: thus D-lysine would be one of the least toxic, given its 4-carbon chain backbone separating the two amino groups. Lack of D-lysine nephrotoxicity on short-term administration is also confirmed in our study, since serum creatinine levels and creatinine clearances (as index of renal involvement) are approximately the same in normal and diabetic animals, regardless of whether the latter were treated from the onset of diabetes with D-lysine or placebo.

Although its chemical reactivity is identical to that of its levo-isomer, the principle of using D-lysine as a non-enzymatic glycation inhibitor is namely: (i) it is not used as a building block for mammalian proteins; (ii) it is apparently not toxic; (iii) it is excreted mainly by glomerular filtration with little or no renal tubular reabsorption [17, 18], and (iv) D-amino acid oxidase activity is very low against D-lysine [17, 26, 27]. These characteristics would explain the finding of significant urinary recovery of D-lysine following i.v. administration reported in past studies [17], also confirmed in this study by the high levels of lysine (which could only be D-lysine) in the urine samples of D-lysine-treated diabetic animals.

Given the characteristics described above, one important question arising concerning the overall effectiveness of D-lysine as a non-enzymatic glycation inhibitor is its physiological handling by cells and tissues. It is accepted that both passive and active transport processes, similar to those working for L-lysine, seem to be functioning for D-lysine, although the rate at which they occur is much slower and is apparently absent at the renal tubular level [28]. This would not affect the activity of the D-amino acid in other tissues, since a steady high blood level would allow enough molecules to react with the free carbonyl group of the aldose glucose, effectively subtracting part of it from reacting with proteins.

The advantages brough about by this type of glycation inhibition are obvious. Other agents, such as aminoguanidine, might reduce the formation of the late by-products of the Maillard reaction very efficiently by reacting irreversibly with early Amadori by-products or by other mechanisms. Yet, in so doing, they might cause the formation, in proteins with long half-lives, of other end-products equally reactive as the ones that they should prevent forming, as has been shown to occur with vitamin C [29]. Dibasic amino acids such as D,L-arginine and L-lysine, which have been shown in vitro to affect the biochemical properties of rat tail tendons by inhibiting non-enzymatic glycation of collagen [30], would be probably actively handled by physiological feed-back pathways. The physiological characteristics and the chemical mode of action of D-lysine would prevent the occurrence of these phenomena and thus would avoid a direct protein involvement. It would also mean that if cessation of treatment was necessary, this could be done without having caused any damage to protein structure and function.

In conclusion, this study has demonstrated that D-lysine treatment in chemically-induced diabetes is capable of reducing the excessive formation of the early products of the Maillard reaction on proteins. Thus, we believe that using long-term pharmacological treatment, the D-amino acid could have an effect on the formation of the late products of the Maillard reaction – as shown in vitro [20] – and that it could potentially delay the appearance of the late pathologies associated with diabetic hyperglycaemia and aging.

*Acknowledgements.* This work was supported by the grant no. 91.00306.40 from the Italian National Research Council (C.N.R., Progetto Finalizzato Invecchiamento, Sottoprogetto 3, Obiettivo 1 pub. code. 933310) and by grants from the International Center for the Study of Diabetes (C.I.S.D.) and from the "Diabetes Endocrinology Metabolism" Foundation, Rome. The authors wish to thank Mr. E. Mazzei and Mr. G. Romani for their skillful technical assistance and medical students Ms G. Iampieri and Ms G. Gagliardi for their enthusiasm. The authors are also indebted to Dr. R. Filippetti (Università Cattolica del Sacro Cuore, Rome) and his staff for their management of the animal care facility.

## References

1. Monnier VM (1989) Toward a Maillard reaction theory of aging. In: Baines JW, Monnier VM (eds) The Maillard reaction in aging, diabetes and nutrition. Alan R. Liss, New York, pp 1–22
2. Monnier VM, Cerami A (1981) Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. Science 211: 491–493
3. Cerami A (1985) Hypothesis: glucose as mediator of aging. J Am Ger Soc 33: 626–634
4. Monnier VM (1990) Nonenzymatic glycosylation, the Maillard reaction and the aging process. J Gerontol 45: B105–B111
5. Brownlee M, Vlassara H, Cerami A (1984) Non-enzymatic glycosylation and the pathogenesis of diabetic complications. Ann Int Med 101: 527–537
6. Sensi M, Pricci F, Andreani D, Di Mario U (1991) Advanced nonenzymatic glycation endproducts (AGE): their relevance to aging and the pathogenesis of late diabetic complications. Diabetes Res 16: 1–9
7. Rendell M, Njerenberg J, Brannan C et al. (1986) Inhibition of glycation of albumin and hemoglobin by acetylation in vitro and in vivo. J Lab Clin Med 108: 286–293
8. Sensi M, Bruno MR, Pozzilli P (1987) In vitro inhibition of nonenzymic glycosylation induced by aspirin. Med Sci Res 15: 99–100
9. Štolba P, Hátle K, Krňáková A, Streda M, Stárka L (1987) Effects of ascorbic acid on nonenzymatic glycation of serum proteins in vitro and in vivo. Diabetologia 30: A585 (Abstract)
10. Davie SJ, Gould BJ, Yudkin JS (1992) Effect of vitamin C on glycosylation of proteins. Diabetes 41: 167–173

11. Bruno MR, Sensi M, Cioccia GP et al. (1988) Inhibition of protein non-enzymatic glycation induced by Bendazac. Diabetes Res 9: 11–14
12. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A (1986) Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. Science 232: 1629–1632
13. Nicholls K, Mandel TE (1989) Advanced glycosylation end-products in experimental murine diabetic nephropathy: effect of islet isografting and of aminoguanidine. Lab Invest 60: 486–491
14. Kumari K, Umar S, Bansal V, Sahib MJ (1991) Inhibition of diabetes-associated complications by nucleophilic compounds. Diabetes 40: 1079–1084
15. Oxlund H, Andreassen TT (1992) Aminoguanidine treatment reduces the increase in collagen stability of rats with experimental diabetes mellitus. Diabetologia 35: 19–25
16. Means GE, Chang MK (1982) Nonenzymatic glycosylation of proteins: structure and function changes. Diabetes 31 [Suppl 3]: 1–4
17. Neuberger A, Sanger F (1944) The metabolism of lysine. Biochem J 38: 119–125
18. Doty J, Eaton AG (1937) The metabolism of lysine: studies on the heat production and blood and urine constituents after administration of D-lysine monohydrochloride to the dog. Am J Phys 119: 296–297
19. Sensi M, Pricci F, De Rossi MG, Morano S, Di Mario U (1989) D-lysine effectively decreases the non-enzymatic glycation of proteins in vitro. Clin Chem 35: 384–387
20. Sensi M, Pricci F, Bellarosa D, Pugliese G, Morano S, Di Mario U (1990) Reduced formation of advanced glycation end products induced by D-lysine: preliminary in vitro observations. In: Belfiore F, Molinatti GM, Reaven GM (eds) Tissue-specific metabolic alterations in diabetes. Frontiers in Diabetes, vol 10. Karger, Basel Munich Paris London New York Tokyo Sydney, pp 158–160
21. Perrett D (1985) Liquid chromatographic separation of amino acids and their derivatives. In: Barrett GC (ed) The chemistry and biochemistry of amino acids. Chapman & Hall, London, pp 426–461
22. Mallia AK, Hermanson GT, Krohn RI, Fujimoto EK, Smith PK (1981) Preparation and use of boronic acid affinity support for separation and quantitation of glycosylated hemoglobins. Anal Letters 14: 649–661
23. Zager AR, Venkatachalam AM (1983) Potentiation of ischemic renal injury by amino acid infusion. Kidney Int 24: 620–625
24. Racusen LC, Finn WF, Whelton A, Solez K (1985) Mechanisms of lysine-induced acute renal failure in rats. Kidney Int 27: 517–522
25. Kaltenback JP, Ganote CE, Carone FA (1979) Renal tubular necrosis induced by compounds structurally related to D-serine. Exp Mol Path 30: 209–211
26. Meister A (1965) Biochemistry of the amino acids. Vol. 1. Academic Press, New York London
27. Nagata Y, Akino T, Ohno K et al. (1987) Free D-amino acids in human plasma in relation to senescence and renal disease. Clin Sci 73: 105–108
28. Schepartz B (1973) Regulation of amino acid metabolism in mammals. Amino acid transport. W.B. Saunders, Philadelphia London Toronto
29. Ortwerth BJ, Olesen PR (1988) Ascorbic acid-induced crosslinking of lens proteins: evidence supporting a Maillard reaction. Biochim Biophys Acta 956: 10–22
30. Menzel EJ, Reihsner R (1991) Alterations of biochemical and biomechanical properties of rat tail tendons caused by non-enzymatic glycation and their inhibition by dibasic amino acids arginine and lysine. Diabetologia 34: 12–16

Received: 15 January 1993
and in revised form: 27 April 1993

Dr. M. Sensi
Cattedra di Medicina Costituzionale ed Endocrinologia
Clinica Medica II
Policlinico Umberto I
Viale del Policlinico
I-00161 Rome
Italy