Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent

Alan R. Hipkiss[a,b,*], Jürgen Michaelis[c], Petros Syrris[b]

[a]*Division of Biomolecular Engineering, CSIRO, 103 Delhi Rd, North Ryde, NSW 2113, Australia*
[b]*Molecular Biology and Biophysics Group, King's College London, Strand, London WC2R 2LS, UK*
[c]*Peptide Technology Ltd., 4-10 Inman Road, Dee Why, NSW 2099, Australia*

Received 14 July 1995

Abstract The dipeptide carnosine (β-alanyl-L-histidine) was readily glycosylated non-enzymatically upon incubation with the sugars glucose, galactose, deoxyribose and the triose dihydroxyacetone. Carnosine inhibited glycation of actyl-Lys-His-amide by dihydroxyacetone and it protected α-crystallin, superoxide dismutase and catalise against glycation and cross-linking mediated by ribose, deoxyribose, dihydroxyacetone, dihydroxyacetone phosphate and fructose. Unlike certain glycated amino acids, glycated carnosine was non-mutagenic. The potential biological and therapeutic significance of these observations are discussed.

Key words: Carnosine; Non-enzymatic glycosylation; Diabetes; Ageing; AGE-product

1. Introduction

Carnosine, a naturally-occurring dipeptide (β-alanyl-L-histidine) first described in 1900 by Gulewitsch and Amiradzibi [1], is found predominantly in post-mitotic tissues (e.g. brain and innervated muscle) of vertebrates. The dipeptide is present in millimolar concentration most likely because of its resistance to cleavage by intracellular proteases and the low activity of specific carnosinases [2]. The biological role of carnosine remains unclear but homeostatic or protective functions have been proposed. The high carnosine concentration found in tissues has a buffering effect at physiological pH [6]. Carnosine is also claimed to decrease oxygen free-radical mediated damage to cellular macromolecules either by chelating divalent cations [3] or scavenging hydroxy radicals with its imidazole moiety [4,5]. Free-radical damage is not the only process to affect the structure of proteins and nucleic acids however. Non-enzymatic glycosylation (glycation), the Maillard reaction in food chemistry [7], involves reaction of amino groups with sugar aldehyde or keto groups to provoke, eventually, cross-linking and advanced glycosylation and products (AGE-products) [8]. Although glycation is slow in vivo, it is of fundamental importance in ageing and in pathological conditions where sugar levels are elevated, e.g. diabetes [9,10] and can result in abnormalities of connective tissue, e.g. collagen cross-linking [11].

Analysis of the preferred glycation sites in proteins shows that the ε-amino groups of lysine residues are primary targets, particularly when in proximity to histidine residues [12]. In a search for stable peptides with long half-lives we found that the amino acid sequence of carnosine is similar to Lys-His, thus having the potential to react with sugars. In this communication we describe the non-enzymatic glycosylation of carnosine and demonstrate its protective action against protein cross-linking.

Materials and methods

Peptides were synthesised by Peptide Technology Ltd., Dee Why, NSW, Australia. All other specialist chemicals and proteins were from Sigma Chemical Co., St. Louis, MO, USA.

Unless otherwise stated, the reaction between peptides and amino acid derivatives with sugars was carried out in phosphate buffered saline, PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) in sealed microcentrifugation vials at 60°C and contained 50 mM peptide and 500 mM sugar. At specified times, samples were withdrawn, diluted 1:20 with water and stored at –20°C prior to analysis by HPLC. The reaction between proteins and sugars was carried out at 37°C in sealed microcentrifuge tubes in 100 mM sodium phosphate buffer pH 7.0 with sodium azide added (0.01%) to prevent microbial growth.

The detection of free amino groups on peptides was performed using a Waters AUTO.OPA system (Waters AUTO.TAG operation manual). In brief, peptides were reacted with *o*-phthalaldehyde and the fluorescent derivate separated by HPLC on a Radical-PAK C_{18} column using a 10% (v/v) to 90% (v/v) methanol gradient as solvent over 15 min. A Waters 470 fluorescence detector set at excitation 340 nm/emission 440 nm was used.

Electrophoresis of proteins (SDS PAGE) was performed by using 4–15% gradient polyacrylamide 'Ready Gels' (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Gels were stained with Coomassie blue [13].

The analysis of mutagenic potential of glycated compounds was performed according to Kim et al. [14]. In brief, D-glucose (1 M) and each of the following: L-carnosine, L-lysine, L-alanine (all 1 M) were dissolved in distilled water, the pH adjusted to 7 and the mixtures heated at 100°C for 80 min. The solutions (50 μl and 100 μl) were evaluated against strain TA 100 of *Salmonella typhimurium* using the plate incorporation method [15] with or without metabolic activation by a standard rat liver microsomal (S-9) preparation. 2-AF and 2-AAF were used as positive controls for the experiments with metabolic stimulation, otherwise sodium azide was included as strain specific positive control.

The spectra of solutions of peptides, amino acids and sugars were obtained using a Cecil C500 spectrophotometer.

3. Results

Spectrophotometric and HPLC analyses provided evidence that carnosine readily reacts with sugars. The non-enzymic

*Corresponding author. Molecular Biology and Biophysics Group, King's College London, Strand, London WC2R 2LS, UK. Fax: (44) (171) 873-2285.

Abbreviations: Ac-, acetyl-; 2AF, 2-aminofluorene; 2AAF, 2-acetamidofluorene; AGE-products, advanced glycosylation end-products; DAHP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; PBS, phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4).

SSDI 0014-5793(95)00849-7

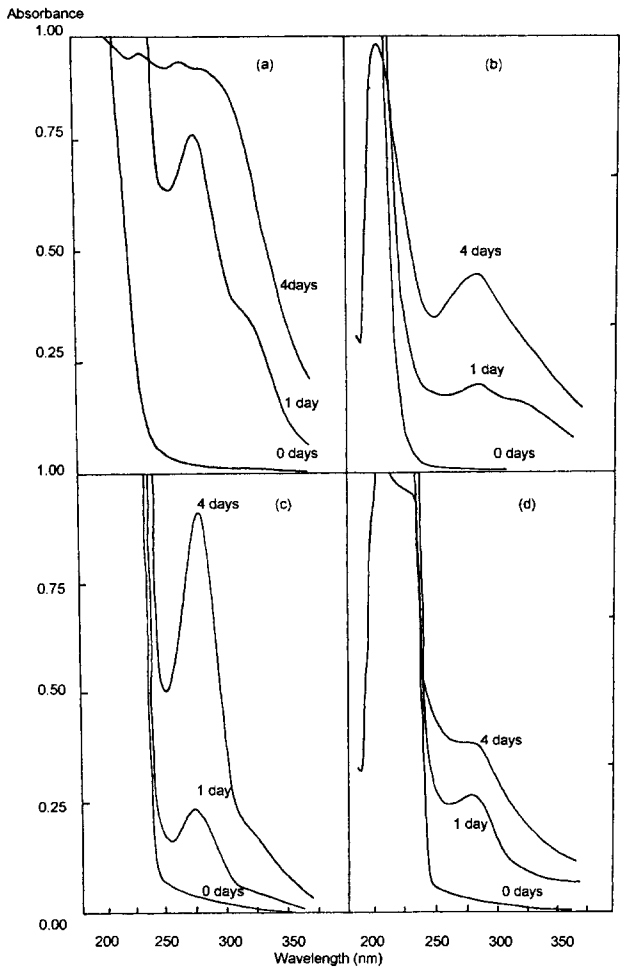


Fig. 1. The effects of incubating carnosine or lysine with deoxyribose or glucose on u.v. absorption. Incubations were carried out in 50 mM sodium phosphate buffer pH 7.0 for 1 or 4 days: concentration of all reactants was 20 mM. (a) lysine + deoxyribose; (b) lysine + glucose; (c) carnosine + deoxyribose; (d) carnosine + glucose.

reaction with proteins, peptides and amino acids with sugars has been extensively studied. The accepted route initially involves the production of a Schiff's base, followed by an Amadori rearrangement and eventually formation of advanced glycosylation end-products (AGE-products) which are frequently brown and cross-linked [7,16]. While the chemistry of these reactions is complex and incompletely characterized, initial evidence for glycation is a change in the u.v. spectrum of the reaction mixture. Typically an increase in absorbance at around 280 nm is detected followed by a smaller increase at around 320 nm wavelength [17]. It is known that lysine is readily glycatable [12,14,17] and Fig. 1 shows the spectral changes that occur following incubation with glucose or deoxyribose. Fig. 1 also shows that carnosine produces similar, though not identical, spectral changes, especially the absorbance peak at around 280 nm. Table 1 shows that both carnosine and lysine produced yellow/brown solutions following prolonged incubations with a glucose concentration in excess of 1 M. In these experiments unphysiological (for mesophiles) temperatures and sugar concentrations were employed to increase reaction rates.

The reaction of carnosine with sugars was also studied by following its disappearance using fluorescence HPLC; the fluorescent agent reacted with amino groups. After 5 h incubation 0.7, 3.6 and 11.9% of the carnosine (initially at 60 mM) had reacted with glucose, galactose and dihydroxyacetone (DHA) (all at 180 mM), respectively, which was in the expected increasing order. Again unphysiological temperatures (60°C) and sugar concentrations were employed for expediency of experimentation. Preferred glycation sites in proteins are amino groups in the vicinity of imidazole functions and/or carboxyl groups [12] e.g. lysyl-histidinyl dipeptides sequences. The dipeptide α-acetyl-lysyl-histidine amide (i.e. with α amino and carboxyl groups blocked as would be the case with Lys-His dipeptide sequences present in a protein) (at 40 mM) was readily glycatable as revealed by the disappearance of its amino group with 16% of it reacting with DHA (80 mM) in 5 h at 60°C. When the two dipeptides (both at 40 mM) were mixed and incubated together with DHA (80 mM), only 4% of the acetyl-Lys-His-amide reacted presumably because of the preferred glycation of carnosine. The dipeptide β-alanyl-glycine was less protective towards acetyl-Lys-His-amide as 11% of the latter reacted in 5 h when these peptides (both at 40 mM) were incubated together with DHA (80 mM). Again elevated temperatures and unphysiological sugar concentrations were employed.

Suger-mediated protein glycation produces cross-linked products as revealed by SDS PAGE by the production of slowly migrating bands. Fig. 2 shows that incubation of α-crystallin with ribose or deoxyribose generates more slowly migrating species i.e. of higher molecular weight, but the additional presence of carnosine inhibited production of the higher molecular weight species in a concentration-dependent manner. Lane 3 (Fig. 2) shows that incubation of the protein with deoxyribose produced a broad band of material which either remained at the top of the gel or migrated very little, and additionally more distinct bands which corresponded to proteins of apparent molecular weights some 3- or 4-times that of the original polypeptide (21,000 Da). Lane 4 shows that 10 mM carnosine decreased production of the very highest molecualr weight material with a corresponding increase in protein of molecular weight around 60,000 Da. Lane 5 shows that 50 mM carnosine prevented the generation of cross-linked crystallin. Lanes 6 and 7 show the effects of incubation of crystallin with ribose for 6 and 9 days, respectively, by the generation of higher molecular weight material especially in lane 7 (9 days). Lanes 8 and 9 clearly show that carnosine prevents the production of the cross-linked protein.

Table 1
Effect of incubating carnosine and amino acids with glucose on u.v. absorption

	Absorption at 400 nM
Glucose	0.175
Glucose + carnosine	8.455
Carnosine	0.041
Glucose + β-alanine	1.240
Glucose + L-lysine	4.170
Glucose + L-arginine	0.469

Incubations carried out in 50 mM sodium phosphate buffer pH 7.0 for 18 h at 50°C. Carnosine and amino acid concentrations used were 500 mM, and glucose employed at 1.39 M.

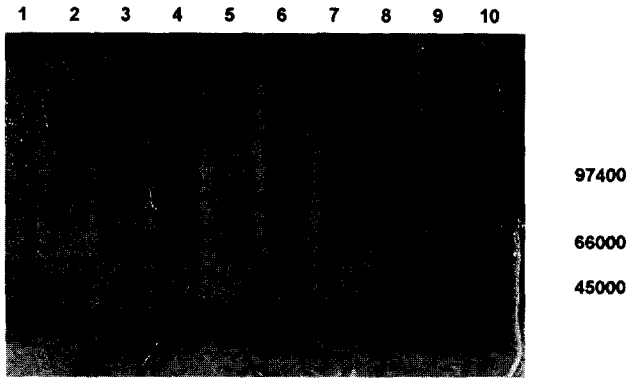
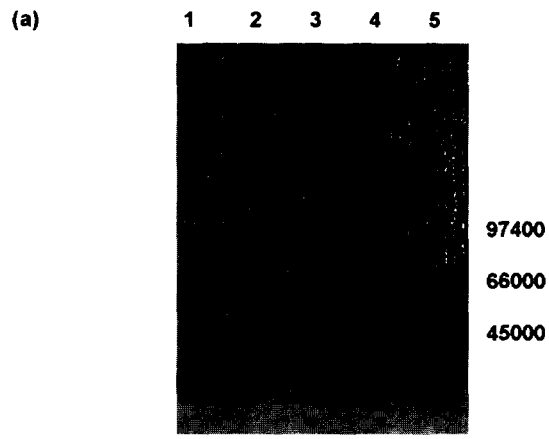
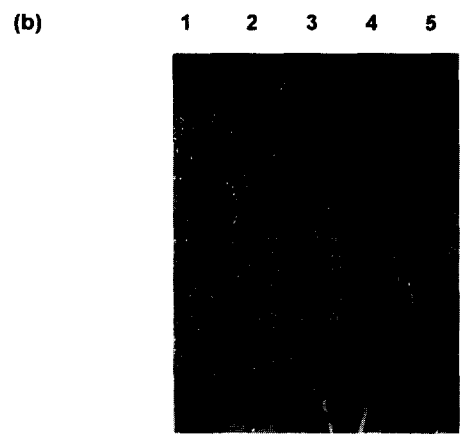


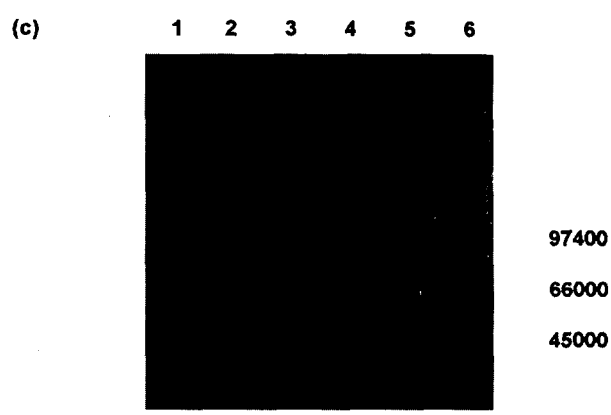
Fig. 2. Inhibition of ribose or deoxyribose-mediated cross-linking of α-crystallin by carnosine. Bovine α-crystallin (100 μg/ml) was incubated in 100 mM sodium phosphate buffer pH 7.0 with 10 mM ribose or deoxyribose with or without carnosine (10–50 mM) at 37°C for 9 days (6 days for lane 6). Lane 1, crystallin; lane 2, crystallin + 25 mM carnosine; lane 3, crystallin + deoxyribose; lane 4, crystallin + deoxyribose + 10 mM carnosine; lane 5, crystallin + deoxyribose + 50 mM carnosine; lane 6, crystallin + ribose (6 days incubation); lane 7, crystallin + ribose; lane 8, crystallin + ribose + 10 mM carnosine; lane 9, crystallin + ribose + 25 mM carnosine; lane 10, marker proteins.

Fig. 3a shows that carnosine protected α-crystallin against cross-linking induced by the highly reactive dihydroxyacetone phosphate (DHAP). Here (lane 2) incubation for 2 days with 1 mM DHAP produces cross-linked crystallin of molecular weights 40,000 and 60,000 Da corresponding to dimers and trimers of the original protein. However, carnosine at 100 mM prevented the generation of the cross-linked protein (lane 4). Fig. 3b illustrates the effect of dihydroxyacetone on superoxide dismutase. Lane 3 shows that the triose induces the production of a rather broad band of material of molecular weight around 45,000 Da approximating to the generation of cross-linked dimers of the protein. Both 25 and 50 mM carnosine prevented the production of the cross-linked polypeptide (lanes 4 and 5, respectively). Fig. 3c shows the effect of fructose on catalase (lane 3) and that carnosine can prevent production of the cross-linked protein (lanes 4 and 5). It should be pointed out that in all experiments carried out using proteins, a temperature of 37°C rather than 50°C or 60°C (as used with lysine and the dipeptides) was employed to decrease the possibility of heat-induced protein denaturation; protection against sugar- mediated protein cross-linking by carnosine also occurred at 50°C however (not shown). Additionally the sugar concentrations were decreased to 10 mM or less in the experiments with proteins, again to improve the physiological appropriateness of these experiments; this necessitated experiments of 2 to 9 days duration. However, protection against sugar-mediated cross-linking using much higher sugar concentrations was again mediated by carnosine (not shown) which affirms the efficacy of the dipeptide as a potential anti-cross-linking agent.

Glycated amino acids such as lysine and arginine are mut-







→

Fig. 3. Inhibition of protein glycation by carnosine. (a) Bovine α-crystallin (100 μg/ml) was incubated in 100 mM sodium phosphate buffer pH 7.0 with 1 mM dihydroxyacetone-phosphate (DHAP) for 2 days at 37°C. Lane 1, crystallin; lane 2, crystallin + DHAP; lane 3, crystallin + DHAP + 10 mM carnosine; lane 4, crystallin + DHAP + 100 mM carnosine; lane 5, marker proteins. (b) Bovine superoxide dismutase (SOD) (100 μg/ml) was incubated in 100 mM sodium phosphate buffer pH 7.0 with 10 mM dihydroxyacetone (DHA) for 2 days at 37°C. Lane 1, SOD; lane 2, SOD + 25 mM carnosine; lane 3, SOD + DHA; lane 4, SOD + DHA + 25 mM carnosine; lane 5, SOD + DHA + 50 mM carnosine. (c) Bovine catalase (CAT) (100 μg/ ml) was incubated in 100 mM sodium phosphate buffer pH 7.0 with 10 mM fructose for 7 days at 37°C. Lane 1, CAT; lane 2, CAT + 25 mM carnosine; lane 3, CAT + fructose; lane 4, CAT + fructose + 50 mM carnosine; lane 5, CAT + fructose + 100 mM carnosine; lane 6, marker proteins.

Table 2
Mutagenic potential of glycated compounds

Compound	Dose (1)	Revertants per plate with TA 100	
		without S-9	with S-9
L-carnosine	250	158 ± 11	149 ± 13
	50	154 ± 14	179 ± 15
L-carnosine glycated	250	142 ± 17	158 ± 19
	50	159 ± 7	167 ± 10
L-lysine glycated	250	277 ± 21	244 ± 13
	50	357 ± 17	553 ± 19
L-alanine glycated	250	145 ± 6	146 ± 9
	50	160 ± 9	181 ± 10
negative control		161 ± 6	188 ± 10
+ azide		>1000	N/A
+ 2AF		N/A	250 ± 33
+ 2AAF		N/A	>500

Salmonella typhimurium TA 100 indicator strain his^- to his^+ reversion system. Data represent the mean number of revertants per plate and their standard deviation for the test solutions and controls with and without metabolic stimulation by rat liver microsomal (S-9) preparation.

agenic [14] as judged by the 'Ames Test' [15]. Other glycated amino acids, such as proline and cysteine, do not exhibit mutagenicity [14]. We investigated the mutagenicity of L-carnosine and the glycated forms of L-carnosine, L-lysine and L-alanine (Table 2). Our data confirm the results of Kim et al. [14] that glycated L-lysine is mutagenic and may therefore be carcinogenic. The activity is slightly enhanced by the rat liver S-9 metabolic activation system. Glycated L-alanine showed no mutagenicity in our experiments and only weak mutagenicity in the earlier work [14]. Both free carnosine and glycated carnosine were not mutagenic. The reason for the difference of the glycated forms of L-carnosine and L-lysine is not known.

4. Discussion

Carnosine has been suggested to have a variety of functions in vitro. Examples are free radical scavenging activity [4,5], chelation of divalent cations [3] and good buffer capacity at neutral pH [6]. The results described in this paper point to the possibility that carnosine has another property. Our results indicate that the dipeptide reacts rapidly with reducing sugars and that the reaction rate is comparable to the lysine-histidine sequence preferentially glycated in proteins in vivo. The structural similarities between carnosine and this amino acid sequence, the dipeptide's ability to decrease glycation of the model peptide Ac-Lys-His-NH_2, and the apparent inhibition of sugar-mediated cross-linking of bovine α-crystallin, superoxide dismutase and catalase by carnosine in vitro would indicate that the dipeptide could possibly play a similar role in vivo. Both superoxide dismutase and catalase are components of the defense system against oxygen free-radicals and may be important in controlling ageing and its related pathologies. That carnosine is associated with neurones which are normally totally dependent on glucose and long-lived is consistent with an anti-ageing role. If carnosine were to be glycated in vivo, the resultant non-mutagenic product would also be consistent with homeostatic functions which preserve protein integrity and decrease the yield of endogenously generated mutagenic agents (i.e. glycated amino acids) upon proteolysis of glycated proteins. However, glycated forms of carnosine of biological origin have yet to be reported. The finding of *N*-carboxymethyllysine, a degradation product of Amadori compounds, in urine [19], and carnosine's structural similarity to preferred glycation sites in proteins suggests that such a search should be undertaken.

Brownlee et al. [20] and Ceriello et al. [21] have observed that glycation of anti-thrombin III (AT-III) interfered with its inhibitory activity towards thrombin and suggested that it is the labile Schiff base adduct to AT-III which decreases its activity during hyperglycaemia. Interestingly, we have observed that carnosine can prevent formation of cross-linked AT-III in model studies (not shown). Similar protective effects by carnosine have also been found when LDL, apoB, fibronectin and laminin were used as target proteins (not shown). The potential anti-glycating property of carnosine suggests that the dipeptide could be considered in treatment of diabetics where glycation is the initial step leading towards important pathological secondary effects. Homocarnosine (γ-amino-butyryl-L-histidine) showed a lower reactivity than carnosine and indicates that minor structural changes influence glycation rates. This may permit the design of carnosine analogues which have different reactivities at selected steps in the glycation process and, together with carnosine, be used as tools for future studies. In vitro glycation of tripeptides has been described [22] but their susceptibility towards peptidases in vivo restricts their potential usefulness. In contrast, carnosine, a β-peptide, is not readily attacked by non-specific peptidases.

The different glycation rates of carnosine by glycose, galactose and dihydroxyacetone we observed reflect the reported differences in sugar reactivities [23]. We have also shown that the fast-reacting sugar, 2-deoxy-D-ribose can glycate carnosine and lysine. We speculate that carnosine could preferentially react with the C_1 of deoxyribose at apurinic sites in DNA, thereby sparing formation of cross-links between nucleoproteins and DNA as well as perhaps facilitating excision repair of the modified DNA. Preliminary studies indicate that carnosine can inhibit DNA/protein cross-linking in model systems (Hipkiss and Farnaud, unpublished). Consistent with this proposal are the observations that carnosine can prevent chromosomal aberrations in Chinese Hamster ovary cells [24] and radiolabelled carnosine is found associated with single stranded DNA fragments following ultraviolet irradiation of human fibroblast in the presence of [^{14}C]carnosine (Hipkiss and Holliday, unpublished observation). Furthermore, the observation that carnosine can delay senescence in cultured human fibroblasts [25] reinforces the proposal that carnosine, usually associated with long-lived cells (i.e. neurones) may possess useful protective properties against the deleterious side effects of glucose, other sugars and oxygen. Furthermore the possibility of an association between glycation and Alzheimer's Disease [26] immediately suggests that the potential use of anti-glycators such as carnosine should be explored.

Acknowledgements: We are grateful to Mr. Methsiri Edirisinghe and Ms. Yen Lam for excellent technical assistance, and to Pharmaceutical Consulting Service, NSW, Australia, for help with the mutagenicity tests. We thank the World Cancer Research Fund for support, the Royal Society for the award of a travel grant to A.R.H. and gratefully acknowledge the hospitality and encouragement of Dr. Robin Holliday (FRS) and his colleagues at CSIRO Molecular Biology Laboratories, North Ryde, Sydney, Australia.

References

[1] Gulewitsch, W. and Amiradzibi, S. (1900) Ber. Dtsch. Chem. Ges. 33, 1902–1903.
[2] Bando, K., Shimotsuji, T., Toyoshima, H. and Hayashi, C. (1984) Ann. Clin. Biochem. 21, 510–514.
[3] Viola, R.E., Hartzell, C.R. and Villafranca, J.J. (1979) J. Inorg. Chem. 10, 293–307.
[4] Boldyrev, A.A., Dupin, A.M., Bunin, A.Y., Babizhaev, M.A. and Severin, S.E. (1987) Biochem. Intern. 15, 1105–1113.
[5] Kohen, R., Yamamoto, Y., Cundy, K.C. and Ames, B. (1988) Proc. Natl. Acad. Sci. USA 85, 3175–3179.
[6] Bate-Smith, E.C. (1938) J. Physiol. 93, 336–343.
[7] Maillard, L.C. (1912) C.R. Acad. Sci. 154, 66–68.
[8] Baynes, J.W. and Monnier, V.M. (1989) The Maillard Reaction in Ageing, Diabetes and Nutrition; Progress in Clinical and Biological Research, Vol. 304, Alan R. Liss, Inc. New York.
[9] Trivelli, L.A., Ranney, H.M. and Hont-Tien, L. (1971) N. Engl. J. Med. 284, 353–357.
[10] Cerami, A. and Koenig, R.J. (1978) Biochem. Sci. April, 73–75.
[11] Monnier, V.M., Vishwanath, V., Frank, K.E., Elmets, C.A., Dauchot, P. and Kohn, R.R. (1986) N. Engl. J. Med. 314, 403–408.
[12] Shilton, B.H. and Walton, D.J. (1991) J. Biol. Chem. 266, 5587–5592.
[13] Neuhoff, R., Stamm, H. and Elble, H. (1985) Electrophoresis 6, 427–448.
[14] Kim, S.B., Kim, I.S., Yeum, D.M. and Park, Y.H. (1991) Mut. Res. 254, 65–69.
[15] Maron, D.M. and Ames, B.N. (1983) Mut. Res. 113, 173–215.
[16] Ledl, F., Beck, J., Sengl, M., Osiander, H., Estendorfer, S., Severin, T. and Huber, B. (1989) in: The Maillard Reaction in Ageing, Diabetes, and Nutrition; Progress in Clinical and Biological Research (Baynes, J.W. and Monnier, V.M., eds.) Vol. 304, pp. 23–42, Alan R. Liss Inc., New York.
[17] Pongor, S. (1984) Proc. Natl. Acad. Sci. USA 81, 2684–2688.
[18] Shin, D.B., Hayase, F. and Kato, H. (1988) Agric. Biol. Chem. 52, 1451–1458.
[19] Ahmed, M.U., Thorpe, S.R. and Baynes, W.J. (1986) J. Biol. Chem. 261, 4889–4894.
[20] Brownlee, M., Vlassara, H. and Cerami, A. (1984) Diabetes 33, 532–535.
[21] Ceriello, A., Giugliano, D., Quatrato, A., Stante, A., Concoli, G., Dello Russo, P. and D'ONofrio, F. (1987) Diab. Metab. 13, 16–19.
[22] Bai, Y., Ueno, H. and Manning, J.M. (1989) J. Prot. Chem. 8, 299–315.
[23] Monnier, V.M., Sell, D.R., Nagaraj, R.H. and Miyata, S. (1991) Gerontology 37, 152–165.
[24] Gille, J.J.P., Pasman, P., van Berkel, C.G.M. and Joenje, H. (1991) Mutagenesis 6, 313–318.
[25] McFarland, G.A. and Holliday, R. (1994) Expl. Cell Res. 212, 167–175.
[26] Yan, S.-D. (1994) Proc. Natl. Acad. Sci. 91, 7787–7791.