CMLS, Cell. Mol. Life Sci. 57 (2000) 747–753
1420-682X/00/050716-14 $ 1.50+0.20/0
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CMLS Cellular and Molecular Life Sciences

Review

A possible new role for the anti-ageing peptide carnosine

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Received 29 November 1999; accepted 27 December 1999

Abstract. The naturally occurring dipeptide carnosine (β-alanyl-L-histidine) is found in surprisingly large amounts in long-lived tissues and can delay ageing in cultured human fibroblasts. Carnosine has been regarded largely as an anti-oxidant and free radical scavenger. More recently, an anti-glycating potential has been discovered whereby carnosine can react with low-molecular-weight compounds that bear carbonyl groups (aldehydes and ketones). Carbonyl groups, arising mostly from the attack of reactive oxygen species and low-molecular-weight aldehydes and ketones, accumulate on proteins during ageing. Here we propose, with supporting evidence, that carnosine can react with protein carbonyl groups to produce protein-carbonyl-carnosine adducts ('carnosinylated' proteins). The various possible cellular fates of the carnosinylated proteins are discussed. These proposals may help explain anti-ageing actions of carnosine and its presence in non-mitotic cells of long-lived mammals.

Key words. Protein; aging; ageing; carbonyl; glycation; proteolysis; lipofuscin; advanced glycosylation end-product.

Carnosine and cell senescence

Carnosine, the dipeptide β-alanyl-L-histidine, has been implicated as an anti-ageing agent [1] because (i) it can delay senescence in cultured human fibroblasts [2, 3], (ii) it can reverse the senescent phenotype in cultured human cells [2, 3] and preserve a juvenile phenotype in cultured rodent cells [4], (iii) it is present in long-lived mammalian tissues at surprisingly high concentrations [5, 6] (up to 20 mM in human muscle) which may decline with age [6] and (iv) tissue levels in mammals appear to correlate directly with the maximum life-span of the species [7, 8]. Many proposals have been made regarding the cellular action of carnosine including a role as an anti-oxidant and oxygen free radical scavenger, a physiological buffer, a histidine source and an immunostimulant [9]. The anti-oxidant and oxygen free radical-scavenging activities of carnosine have been demonstrated in many studies [10–12]. However, other anti-oxidants do not have the anti-ageing actions on cultured human fibroblasts described by McFarland and Holliday suggesting that additional properties of the dipeptide are responsible for, or contribute to, these effects [1, 7]. More recent evidence shows that carnosine can react with low-molecular-weight aldehydes and ketones indicating that it might also act as a naturally occurring anti-glycating agent [13–15]. We now propose yet another possible mechanism of action of carnosine which may contribute to its anti-ageing properties, namely an ability to react with carbonyl groups present on proteins.

Carnosine and glycating agents

Glycation (also called non-enzymic glycosylation) is the process whereby reducing sugars react with protein

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amino groups to generate initially a Schiff's base, then an Amadori product and finally advanced glycosylation end-products (AGEs) [16]. The chemically complex highly cross-linked AGE structures are often deleterious to cells because they can provoke a hyperoxic response in those which bear appropriate receptors (scavenging or RAGEs) [17]. The structure of carnosine closely resembles that of preferred glycation sites in proteins, that is, a target amino group with proximal imidazole and carboxyl groups. It can react with many potential glycating agents thereby inhibiting their ability to react with and modify polypeptides, as shown by our studies of protein cross-linking in the presence of glucose, fructose, ribose, deoxyribose, dihydroxyacetone, glyceraldehyde, malondialdehyde and methylglyoxal [13–15, 18–20]. Such anti-glycating activity has recently been confirmed by others [21–24]. Carnosine has been shown to protect cells (cultured human fibroblasts and lymphocytes, cultured rat brain endothelial cells and Chinese hamster ovary cells) against the deleterious effects of acetaldehyde and malondialdehyde (a lipid peroxidation end-product), presumably due to its ability to react with these toxic aldehydes [15, 18, 25]. Furthermore, the dipeptide also ameliorated the toxicity of a low-molecular-weight AGE formed between lysine and deoxyribose [18] and inhibited DNA-protein cross-linking induced by acetaldehyde and formaldehyde [18]. Studies using a model system also showed that carnosine inhibited modification of a protein by the AGE formed from the reaction of lysine with methylglyoxal [20].

Table 1. The binding of radiolabelled carnosine and lysine to methylglyoxal-treated and untreated ovalbumin.

		Radioactivity bound to protein (cpm)	
		^{14}C-carnosine	^{14}C-lysine
Experiment I	MG-treated ovalbumin	833 (100)	821 (100)
	untreated ovalbumin	47 (5.6)	8 (0.9)
Experiment II	MG-treated ovalbumin	626 (100)	
	plus 50 mM lysine	142 (22.7)	
Experiment III	MG-treated ovalbumin	568 (100)	
	plus 50 mM ester	213 (37.5)	
Experiment IV	MG-treated ovalbumin		508 (100)
	plus 50 mM carnosine		152 (29.9)

Ovalbumin (10 mg/ml) was incubated at 37 °C in the presence or absence of methylglyoxal (MG, 10 mM) for 1 week in experiment I and 2 weeks in experiments II–IV. Following extensive dialysis, the treated and untreated ovalbumins were incubated for a further 3 weeks in the presence of either ^{14}C-carnosine (5.8 nCi/ml) or ^{14}C-lysine (5.5 nCi/ml) plus or minus unlabelled lysine [or N-(α)-acetylglycyl-lysine methyl ester] or carnosine, respectively. The protein was then precipitated with trichloracetic acid, the precipitate extensively washed and then redissolved in NaOH and counted in a liquid scintillation spectrometer. The results are presented as cpm/ml of incubate, and as a percentage (in parentheses) of the radioactivity bound to the protein in the absence of any potential competitor (i.e. binding in absence of inhibitor in each experiment = 100%).

Carnosine and protein carbonyls

AGEs and their precursors frequently contain reactive carbonyl groups which can be generated by the actions of oxygen free radicals and related species (reactive oxygen species) as well as aldehydes/ketones on amino acid side chains [26]. Indeed, the accumulation of aberrant protein molecules bearing carbonyl groups is a common molecular signal of ageing or senescence [26, 27]. Little is known about how cells defend themselves against protein carbonyls except that some oxidized proteins may be selectively degraded [28, 29], but extensively modified polypeptides appear to be resistant to intracellular proteolysis [29, 30]. We therefore sought to determine whether carnosine might also have a protective role by interacting with AGEs and polypeptides bearing carbonyl groups thus preventing cross-linking reactions which are thought to occur with other (normal) molecules.

In our recent attempts to address this possibility [unpublished observations], carbonyl groups were generated in ovalbumin by the reaction with methylglyoxal, a physiologically relevant glycating agent [31]. [^{14}C]-labelled carnosine was incubated with the treated protein, from which unreacted methylglyoxal had been removed, for various periods of time. Free carnosine is acid-soluble but gradually some of the radiolabel became incorporated into the acid-precipitable protein fraction, which did not occur when control untreated ovalbumin was employed (table 1). This result is consistent with our proposition that carnosine reacts with the carbonyl groups present on the modified polypeptide. Further support for this idea came from the demonstration that the dipeptide accelerated the loss of carbonyl groups (measured by dinitrophenylhydrazine reactivity) from the modified protein (fig. 1). Lysine, which is known to react with carbonyl groups in proteins, also accelerated this loss (fig. 1) and inhibited the radiolabelling of the methylglyoxal-treated ovalbumin incubated with [^{14}C]-carnosine (table 1). Additionally N-(α)-acetylglycyl-lysine methyl ester, which more closely resembles lysine residues present in polypeptide chains, inhibited the reaction of [^{14}C]-carnosine with the treated protein. Conversely, carnosine inhibited the reaction of [^{14}C]-

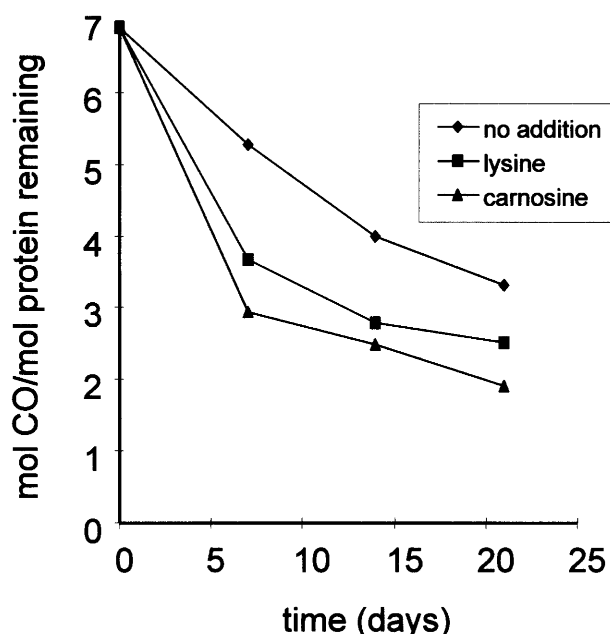

Figure 1. Loss of carbonyl groups in methylglyoxal-treated ovalbumin in the presence or absence of carnosine/lysine. Ovalbumin (10 mg/ml), incubated for 2 weeks at 37 °C with 10 mM methylglyoxal, was extensively dialysed before further incubation in the presence or absence of either 50 mM carnosine or lysine at 37 °C. Carbonyl groups remaining after various periods of time were measured by diphenylhydrazine reactivity and the molar ratio of CO groups to protein are presented.

lysine with the methylglyoxal-treated protein (table 1). The reaction of carnosine with protein carbonyls might possibly inhibit further cross-linking to normal proteins. We tested this possibility and found that carnosine did indeed inhibit the cross-linking activity of the methylglyoxal-treated ovalbumin towards the normal protein α-crystallin (fig. 2). We suggest from these observations that not only can carnosine react with protein carbonyls but it can also moderate their deleterious action towards other molecules. Protein carbonyls and AGEs are found in increased amounts in ageing tissues, particularly in uncontrolled diabetics where they are responsible for many of the secondary complications [16, 17, 32] and, therefore, carnosine (or related structures) could be of clinical interest in the control of such processes. The term 'carnosinylation' has been coined to describe the process in which carnosine reacts with protein carbonyl groups.

Possible biological fates of 'carnosinylated' proteins

We do not know whether carnosine reacts with carbonyl groups present on aged proteins in cultured cells or in vivo, although experiments are planned to test this idea. If carnosine does react intracellularly as predicted, then it is of interest to consider the fate of the postulated protein-carbonyl-carnosine adduct. There are a number of possibilities (see fig. 3). First it is noteworthy that lipofuscin, the fluorescent age pigment, is found primarily in carnosine-rich tissues such as nerve, muscle and brain [33]. Thus it is possible that the reaction of carnosine with the protein carbonyls might create a relatively inert form of lipofuscin [33, 34]. Our preliminary studies [unpublished data] show that the reaction of carnosine with a protein glycated with acrolein (another source of protein carbonyls) [35] induces a change in the fluorescent properties of the protein-acrolein adduct. A second possibility is that the carnosinylated protein might be a form of AGE that is expelled from the cell to be taken up by appropriate receptors (e.g. RAGEs) and subsequently degraded by the lysosomes [17]. A third alternative is that carnosinylation of an aged protein is a signal for its selective degradation via the proteasome system of multicatalytic proteases. Recent studies have shown, however, that highly oxidized proteins can inhibit proteasome function as well as cell division when added to cultured human fibroblasts [28, 29]. Hence, it will be interesting to find out if carnosinylation of highly oxidized proteins can (i) suppress their inhibitory effects and (ii) facilitate proteolysis of the aberrant polypeptides. A fourth possibility is that the reaction with carnosine might enable ubiquitination of the protein at preserved polypeptide amino groups, or should the amino group of any attached carnosine become available. If so, then one would again expect to observe the degradation of the aged protein via the proteasome system.

Further experiments to examine these ideas are necessary. However, preliminary experiments show that proteolysis of a slow-turnover fraction is stimulated when fibroblasts are cultured with 20 mM carnosine particularly in cells approaching their maximal division potential [15], a result consistent with carnosine affecting proteolysis. This concept is also supported by the observation that carnosine induces a more juvenile phenotype when added to senescent cells which is accompanied by the loss of much granular material [2, 3]. Obvious questions which need to be addressed are: (i) is it possible to observe the specific proteolysis of carnosinylated proteins following addition of carnosine to senescent cells? (ii) does a transient attachment of carnosine to accumulated aberrant protein occur? and (iii) does the addition of proteasome or lysosomal protease inhibitors stimulate accumulation of carnosinylated proteins?

Wider implications for the carbonyl-binding potential of carnosine

If the suggestions that carnosine is an anti-glycating agent for proteins and that it determines the fate of

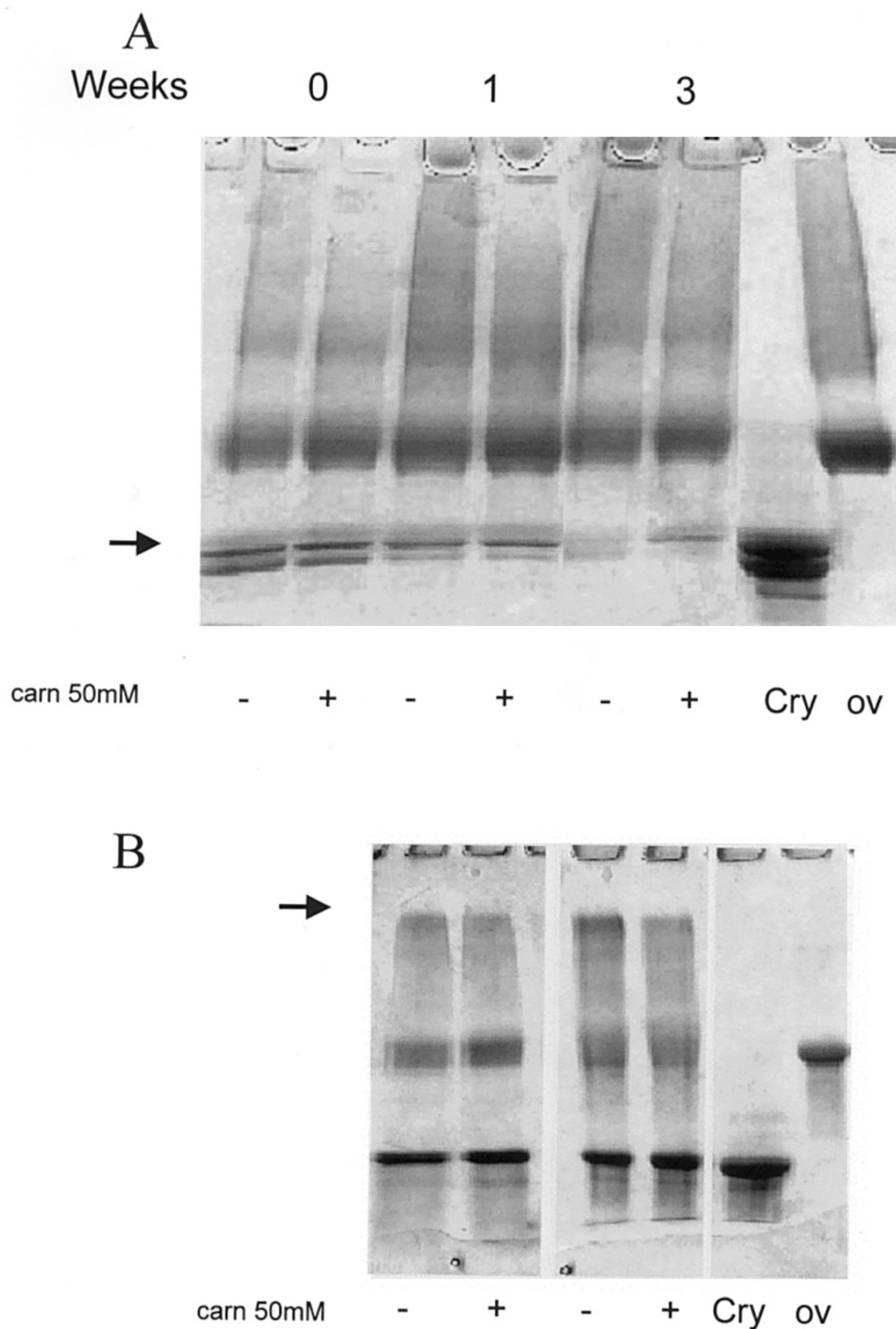

Figure 2. Analysis of methylglyoxal-treated ovalbumin cross-linking to α-crystallin in the presence and absence of carnosine by sodium docecylsulphate (SDS)-polyacrylamide gel eletrophoresis. Dialysed methylglyoxal-treated ovalbumin (5 mg/ml) (ov) was incubated at 37 °C in the presence (+) or absence (−) of 50 mM carnosine (carn) with either 0.4 mg/ml (*A*) or 2.5 mg/ml (*B*) α-crystallin (Cry) for 0, 1 and 3 weeks. SDS-polyacrylamide gel electrophoresis was carried out on Bio-Rad 4–20% Tris-HCl Ready gels. (*A*) Illustrates the loss of α-crystallin (arrow). (*B*) Shows the formation of high-molecular-weight cross-linked material (arrow).

aged proteins are correct, then this opens new avenues in molecular gerontology. The possibility that macromolecules other than proteins may also be a source of deleterious carbonyls should not be neglected. The serine and ethanolamine present in aminolipids are already known to be targets for oxidative attack and the products of such reactions may react with other amino-lipid molecules and proteins present in cell membranes [16]. Therefore, carnosine might conceivably react with lipid carbonyls as well, thereby protecting membranes

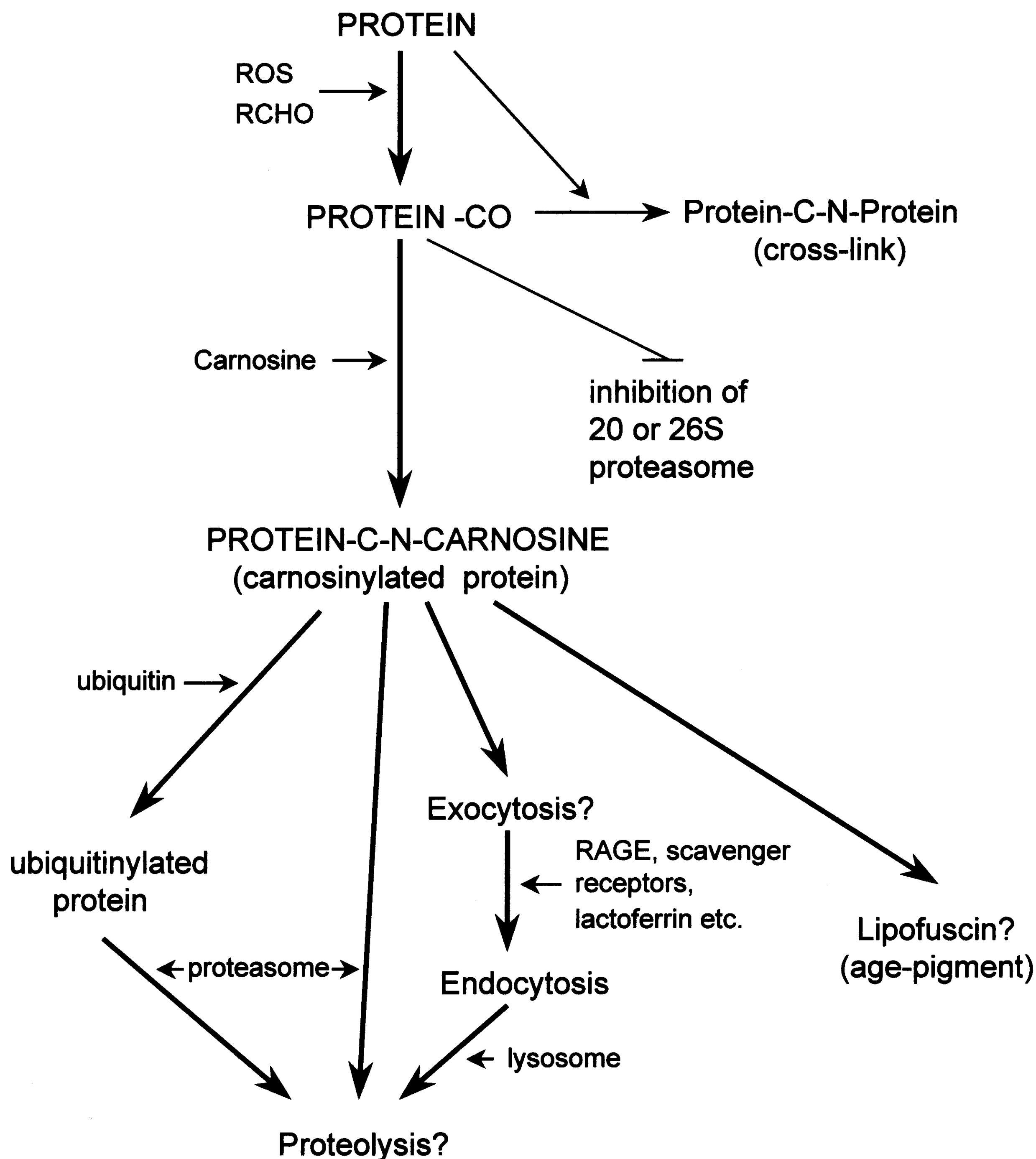

Figure 3. Schematic representation of formation and possible fates of 'carnosinylated' proteins. Reactions between carnosine and reactive oxygen species, aldehydes and ketones are omitted for clarity. ROS, reactive oxygen species; RCHO, reactive aldehydes and ketones including aldose and ketose sugars; protein-CO, protein carbonyl.

against lipid-protein cross-linking. The release of purines from DNA during spontaneous depurination, a well-recognized common age-related change to the genetic material, is another source of reactive aldehydes [36]. Additionally, the cleavage of the glycosidic bond between the base and the deoxyribose following base-excision repair of oxidatively or otherwise damaged purines and pyrimidines exposes the reactive aldehyde group of the sugar moiety [37]. The subsequent fate of this sugar is uncertain but there has been the proposition that an even more reactive bicarbonyl compound is generated [38]. Given the reactivity of carnosine towards the bicarbonyl malondialdehyde [19], the dipeptide may quite possibly perform a useful scavenging function which would accompany DNA repair when carbonyl-bearing products are generated. Indeed, carnosine has been shown to preserve chromosomal integrity in cultured cells [39] and to prevent the formation of protein-DNA cross-links in model systems [18].

The carnosinase paradox

A major paradox in any attempt to understand the functions and mechanisms of action of carnosine is the presence of carnosinase which cleaves the dipeptide into its constituents, β-alanine and histidine, both in cells and serum [40]. The necessity for this enzyme activity is unclear given the almost non-toxic nature of carnosine and the toxicity of free histidine. Perhaps carnosine possesses some deleterious properties not hitherto recognised. Alternatively, the active agent in many of the functions of carnosine (e.g. carbonyl binding) in vivo may be histidine (and β-alanine) and not the dipeptide. Consistent with this idea, the reaction product of histidine and formaldehyde, namely spinacine, can be found in human urine.

Conclusion

Carnosine clearly has a number of homeostatic functions including scavenging of oxygen free radicals and low-molecular-weight aldehydes/ketones. We now propose that the ability of carnosine to delay senescence in cultured cells may also be related to its affinity for carbonyl groups present in polypeptides damaged by glycation and oxidative events. Some concern may be raised that yet more possible mechanisms for the action of carnosine are suggested here when so many have already been proposed during the almost 100 years since its discovery. However, if the dipeptide is an anti-ageing agent then some, if not considerable, pluripotency might be expected given the multifactorial nature of ageing.

Acknowledgments. The support of the Wellcome Trust is gratefully acknowledged.

1 Hipkiss A. R. (1998) Carnosine, a protective anti-ageing peptide? Int. J. Biochem. Cell Biol. **30:** 863–868
2 McFarland G. A. and Holliday R. (1994) Retardation of the senescence of cultured human diploid fibroblasts by carnosine. Exp. Cell Res. **212:** 167–175
3 McFarland G. A. and Holliday R. (1999) Further evidence for the rejuvenating effects of the dipeptide L-carnosine on cultured human diploid fibroblasts. Exp. Gerontol. **34:** 35–45
4 Kantha S. S., Wada S., Tanaka H., Takeuchi M., Watabe S. and Ochi H. (1996) Carnosine sustains the retention of cell morphology in continuous fibroblast culture subjected to nutritional insult. Biochem. Biophys. Res. Commun. **223:** 278–282
5 Johnson P., Fedyna J. S., Schindzielorz A., Smith C. M. and Kasvinshy P. J. (1982) Regulation of muscle phosphorylase activity by carnosine and anserine. Biochem. Biophys. Res. Commun. **109:** 769–775
6 Stuenenburg H. J. and Kunze K. (1999) Concentration of free carnosine (a putative membrane-protective antioxidant) in human muscle biopsies and rat muscles. Arch. Gerontol. Geriatrics **29:** 107–113
7 Hipkiss A. R., Holliday R., McFarland G. and Michaelis J. (1993) Carnosine and senescence. Lifespan **4:** 1–3
8 Munch G., Thome J., Foley P., Schinzel R. and Riederer P. (1997) Advanced glycation end products in ageing and Alzheimer's disease. Brain Res. Rev. **23:** 134–143
9 Quinn P. R., Boldyrev A. A. and Formazuyk V. E. (1992) Carnosine: its properties, functions and potential therapeutic applications. Mol. Aspects Med. **13:** 379–444
10 Kohen R., Yamamoto Y., Cundy K. C and Ames B. N. (1988) Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. Proc. Natl. Acad. Sci. USA. **95:** 2175–2179
11 Boldyrev A. A., Formazyuk V. E. and Sergienko V. I. (1994) Biological significance of histidine-containing dipeptides with special reference to carnosine: chemistry, distribution, metabolism and medical applications. Sov. Sci. Rev. D. Physicochem. Biol. **13:** 1–60
12 Aruoma O. I., Laughton M. J. and Halliwell B. (1989) Carnosine, homocarnosine and anserine: could they act as anti-oxidants in vivo? Biochem. J. **264:** 863–869
13 Hipkiss A. R., Michaelis J., Syrris P., Kumar S. and Lam Y. (1994) Carnosine protects proteins against in vitro glycation and cross-linking. Biochem. Soc. Trans. **22:** 399S
14 Hipkiss A. R., Michaelis J. and Syrris P. (1995) Non-enzymic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. FEBS Lett. **371:** 81–85
15 Hipkiss A. R., Michaelis J., Syrris P. and Dreimanis M. (1995) Strategies for the extension of human life span. Perspect. Hum. Biol. **1:** 59–70
16 Bierhaus A., Hofman M., Ziegler R. and Nawroth P. P. (1998) AGEs and their interactions with AGE receptors in vascular disease and diabetes mellitus. 1. The AGE concept. Cardiovasc. Res. **37:** 586–600
17 Thornalley P. J. (1998) Cell activation by glycated proteins: AGE receptors, receptor recognition factors and functional classification of AGEs. Cell Mol. Biol. **44:** 1013–1023
18 Hipkiss A. R., Preston J. E., Himsworth D. T. M., Worthington V. C., Keown M., Michaelis J. et al. (1998) Pluripotent protective effects of carnosine, a naturally-occurring dipeptide. Ann. N.Y. Acad. Sci. **854:** 37–53
19 Hipkiss A. R., Worthington V. C., Himsworth D. T. M. and Herwig W. (1997) Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite. Biochim. Biophys. Acta **1380:** 46–54
20 Hipkiss A. R. and Chana H. (1998) Carnosine protects proteins against methylglyoxal-mediated modifications. Biochem. Biophys. Res. Commun. **248:** 28–32

21 Lee B. J., Kang K. S., Nam S. Y., Park J. H., Lee Y. S., Yun Y. W. et al. (1999) Effects of carnosine and related compounds on monosaccharide autoxidation and H_2O_2 formation. Korean J. Physiol. Pharmacol. **3:** 251–261
22 Swearengin T. A., Fitzgerald C. and Seidler N. W. (1999) Carnosine prevents glyceraldehyde 3-phosphate-mediated inhibition of aspartate aminotransferase. Mol. Toxicol. **73:** 307–309
23 Vinson J. A. and Howard T. B. (1996) Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. Nutr. Biochem. **7:** 659–663
24 Kuleva N. V. and Kovalenko Z. S. (1997) Change in the functional properties of actin by its glycation in vitro. Biochemistry [Moscow] **62:** 1119–1123
25 Hipkiss A. R., Preston J. E., Himsworth D. T. M., Worthington V. C. and Abbot N. J. (1997) Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells. Neurosci. Lett. **238:** 135–138
26 Stadtman E. R. (1992) Protein oxidation and aging. Science **257:** 1220–1224
27 Goto S., Nakamura A., Radak Z., Nakamoto H., Takahashi R., Yasuda K. et al. (1999) Carbonylated proteins in aging and exercise: immunoblot approaches. Mech. Aging Dev. **107:** 245–253
28 Sitte N., Merker K. and Grune T. (1998) Proteosome-dependent degradation of oxidised proteins in MRC-5 fibroblasts. FEBS Lett. **440:** 399–402
29 Reinheckel T., Sitte N., Ullrich O., Kuckelkorn U., Davies K. J. A. and Grune T. (1998) Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochem. J. **335:** 637–642
30 Grant A. J., Jessup W. and Dean R. T. (1992) Accelerated endocytosis and incomplete catabolism of radical-damaged protein. Biochim. Biophys. Acta **1134:** 203–209
31 Chaplen F. W. R. (1998) Incidence and potential implications of the toxic metabolite methylglyoxal in cell culture: a review. Cytotechnology **26:** 173–183
32 Baynes J. W. and Monnier V. M. (1989) The Maillard Reaction in Aging, Diabetes and Medicine. Liss, New York
33 Yin D. Z. (1995) Studies on age pigments evolving into a new theory of biological aging. Gerontology **41:** 159–170
34 Brunk U. T., Jones C. B. and Sohal R. S. (1992) A novel hypothesis of lipofuscinogensis and cellular aging based on interactions between oxidative stress and autophagocytosis. Mutat. Res. **275:** 395–404
35 Uchida K., Kanematsu M., Sakai K., Matsuda T., Hattori N., Mizuno Y. et al. (1998) Protein-bound acrolein: potential markers for oxidative stress. Proc. Natl. Acad. Sci. USA **95:** 4882–4887
36 Lindahl T. (1993) The instability and decay of the primary structure of DNA. Nature **362:** 709–715
37 Sancar A. (1995) Excision repair in mammalian cells. J. Biol. Chem. **270:** 15915–15918
38 Nash H. M., Bruner S. D., Scharer O. D., Kawate T., Addona T. A., Spooner E. et al. (1996) Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. Curr. Biol. **6:** 968–980
39 Gille J. J. P., Pasman P., Berkel C. G. M. van and Joenje H. (1991) Effects of antioxidants on hyperoxia-induced chromosomal breakage in Chinese hamster ovary cells: protection by carnosine. Mutagenesis **6:** 313–318
40 Kunze H., Kleinkauf H. and Bauer K. (1986) Characterization of two carnosine-degrading enzymes from rat brain: partial purification of a carnosinase and a β-alanyl-arginine hydrolase. Eur. J. Biochem. **160:** 605–613