Carnosine, homocarnosine and anserine: could they act as antioxidants *in vivo*?

Okezie I. ARUOMA, Miranda J. LAUGHTON and Barry HALLIWELL

Department of Biochemistry, University of London King's College, Strand Campus, London WC2R 2LS, U.K.

Carnosine, homocarnosine and anserine have been proposed to act as antioxidants *in vivo*. Our studies show that all three compounds are good scavengers of the hydroxyl radical ($\cdot\text{OH}$) but that none of them can react with superoxide radical, hydrogen peroxide or hypochlorous acid at biologically significant rates. None of them can bind iron ions in ways that interfere with 'site-specific' iron-dependent radical damage to the sugar deoxyribose, nor can they restrict the availability of Cu^{2+} to phenanthroline. Homocarnosine has no effect on iron ion-dependent lipid peroxidation; carnosine and anserine have weak inhibitory effects when used at high concentrations in some (but not all) assay systems. However, the ability of these compounds to interfere with a commonly used version of the thiobarbituric acid (TBA) test may have led to an overestimate of their ability to inhibit lipid peroxidation in some previous studies. By contrast, histidine stimulated iron ion-dependent lipid peroxidation. It is concluded that, because of the high concentrations present *in vivo*, carnosine and anserine could conceivably act as physiological antioxidants by scavenging $\cdot\text{OH}$, but that they do not have a broad spectrum of antioxidant activity, and their ability to inhibit lipid peroxidation is not well established. It may be that they have a function other than antioxidant protection (e.g. buffering), but that they are safer to accumulate than histidine, which has a marked pro-oxidant action upon iron ion-dependent lipid peroxidation. The inability of homocarnosine to react with HOCl , interfere with the TBA test or affect lipid peroxidation systems in the same way as carnosine is surprising in view of the apparent structural similarity between these two molecules.

INTRODUCTION

Carnosine (β -alanyl-L-histidine) and related compounds such as anserine (β -alanyl-L-methyl-L-histidine) and homocarnosine (γ -amino-butryl-L-histidine) are present at millimolar concentrations in several mammalian tissues, including skeletal muscle and brain [1–4], although there are interesting differences in their tissue distributions [3,4]. The structures of these compounds are shown in Fig. 1. Their precise metabolic function is unknown [5]; suggestions have included the possibility that they buffer metabolically produced acids (presumably a function of the imidazole ring) and that they might act as antioxidants *in vivo* [1,2,6]. Thus Kohn *et al.* [1] reported that carnosine decreased the rate of oxidation of linoleic acid by peroxy radicals and inhibited hydroxylation of deoxyguanosine by an ascorbic acid/ Cu^{2+} mixture, apparently by binding Cu^{2+} . Dahl *et al.* [6] found that carnosine is a powerful quencher of singlet oxygen. Boldyrev *et al.* (reviewed in [2]) claimed that carnosine inhibits lipid peroxidation in membranes.

However, no complete characterization of the antioxidant activity of carnosine has been reported. In the present paper, we describe a detailed examination of the ability of carnosine, homocarnosine and anserine to react with four species: superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$) and hypochlorous acid (HOCl). Superoxide and H_2O_2 are formed during normal aerobic metabolism [7]. In the presence of suitable transition metal ions, O_2^- and H_2O_2 can interact to form $\cdot\text{OH}$; iron ions seem to be the most likely catalysts

of this interaction *in vivo* [7–9]. The ability of carnosine, homocarnosine and anserine to bind iron ions in ways that affect the ability of these ions to generate $\cdot\text{OH}$ from H_2O_2 has therefore also been investigated. HOCl is formed by myeloperoxidase and contributes to tissue damage at sites of inflammation (reviewed in [10]). It is therefore interesting to see if HOCl could be scavenged by carnosine, homocarnosine or anserine. We have also examined in detail the ability of carnosine, homocarnosine and anserine to inhibit peroxidation of lipids, using liposomes and rat-liver microsomes as substrates for peroxidation. Lastly, some studies on the ability of these three compounds to bind Cu^{2+} have been performed.

MATERIALS AND METHODS

L-Carnosine, L-homocarnosine sulphate, L-anserine nitrate, β -alanine and L-histidine were from Sigma. Solutions were carefully adjusted to pH 7.4 before use. Other reagents were of the highest quality available from Sigma or from BDH Chemicals Ltd. α_1 -Antiproteinase (α_1 -AP) was Sigma type A9024. Elastase and α_1 -AP were assayed as described in [11], HOCl being produced immediately before use by adjusting NaOCl to pH 6.2 with dilute H_2SO_4 [11]. Deoxyribose degradation in the presence of ascorbate, H_2O_2 and FeCl_3 ($\pm$ EDTA) [12] was measured by a thiobarbituric acid (TBA) test; at the end of the incubation, 1 ml of 1% (w/v) TBA in 0.05 M-NaOH and 1 ml of 2.8% (w/v) trichloroacetic acid were added, and the tubes were heated at 100 °C for 15 min on

Abbreviations used: α_1 -AP, α_1 -antiproteinase; TBA, thiobarbituric acid; NBT, Nitro Blue Tetrazolium; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole.

a heating block. After cooling, A_{532} was measured [12]. Generation of O_2^- by the hypoxanthine-xanthine oxidase system was carried out essentially as in [13]. Reaction mixtures (final vol. 3 ml) contained 0.1 ml of 30 mM-EDTA, 10 μ l of 30 mM-hypoxanthine, 100 μ l of 3 mM-cytochrome *c* or 3 mM-Nitro-Blue Tetrazolium (NBT) and 50 mM (final concentration) KH_2PO_4/KOH buffer, pH 7.4. The reaction was started by adding 0.2 ml of xanthine oxidase (Sigma) (freshly diluted in 50 mM- KH_2PO_4/KOH buffer, pH 7.4, to 1 unit/ml) and rates of NBT or cytochrome *c* reduction were measured at 560 or 550 nm respectively at 25 °C. H_2O_2 was measured by the formation of a brown colour (recorded at 436 nm) in reaction mixtures, of final volume 1 ml, containing 0.15 M- KH_2PO_4/KOH buffer, pH 7.4, 50 μ l of guaiacol solution (100 μ l pure guaiacol mixed with 50 ml of water) and 10 μ l of horseradish peroxidase (Sigma type VI; 5 mg/ml in buffer). The rate of absorbance change at 436 nm is proportional to the concentration of added H_2O_2 . Carnosine, homocarnosine and anserine were incubated at concentrations up to 9.6 mM with 3.5–10.0 mM- H_2O_2 for 30 min at 25 °C. Aliquots were then taken and assayed for remaining H_2O_2 by using the peroxidase system. H_2O_2 was also measured by titration with 1 mM- $KMnO_4$ solution after solutions were made 0.1 M in H_2SO_4 . The end-point of the titration was when the purple colour of the $KMnO_4$ was no longer discharged.

Ox-brain phospholipid liposomes were prepared as in [14], and rat liver microsomes as in [15]. Peroxidation of liposomes was studied at pH 7.4 in the presence of 100 μ M final concentrations of ferrous ammonium sulphate, or $FeCl_3$ and ascorbic acid (both 100 μ M), as in [15]. Solutions of iron salts or ascorbic acid were freshly made up in N_2 -sparged water immediately before use. Tubes were incubated at 37 °C for 2 h, unless otherwise stated. Lipid peroxidation was measured by a TBA test. At the end of the incubation, 0.5 ml of 1% (w/v) TBA in 0.05 M-NaOH and 0.5 ml of 25% (v/v) HCl were added and tubes were heated at 100 °C for 15 min [15]. Some studies were also performed in which the HCl was replaced by trichloroacetic acid, as used in the deoxyribose assay described previously. Peroxidation was also measured by a fluorescence method [14,15], by measuring the uptake of O_2 [16] using a Hansatech O_2 electrode maintained at 25 °C, or by diene conjugation after extraction of lipids into chloroform/methanol [16]. Peroxidation of microsomes was studied as in [15] using 0.1–0.5 mg of microsomal protein per 2 ml of reaction mixture. Peroxidation was started by adding 100 μ M ferrous ammonium sulphate, or 100 μ M- $FeCl_3$ plus 100 μ M-ascorbic acid, or 100 μ M- Fe^{2+} plus 100 μ M- Fe^{3+} , or 100 μ M- Fe^{2+} -ADP, or 100 μ M- Fe^{3+} -ADP plus 400 μ M-NADPH. Iron salts and ADP were pre-mixed before addition to the reaction mixture. Peroxidation was measured by the methods described previously. KNO_3 or Na_2SO_4 at concentrations equal to those of homocarnosine or anserine had no effect on peroxidation in any system examined. The phenanthroline assay was carried out exactly as in [17].

RESULTS

Action on superoxide

A mixture of hypoxanthine and xanthine oxidase at pH 7.4 generates O_2^- , which can be detected by its ability

Fig. 1. Structures of carnosine (a), homocarnosine (b) and anserine (c)

to reduce ferricytochrome *c* [18] or NBT [19]. Any added compound that is itself able to react with O_2^- should decrease the rate of reduction of these substances. Carnosine, homocarnosine and anserine, tested at concentrations of up to 4 mM, had no effect on the rate of reduction of 100 μ M-NBT or 100 μ M-cytochrome *c* by O_2^- (none of the tested substances themselves reduced cytochrome *c* or NBT).

Under our reaction conditions, cytochrome *c* reacts with O_2^- with a second-order rate constant of $2.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ [20], and NBT reacts with a rate constant of about $6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ [21]. The inability of carnosine, homocarnosine and anserine, at concentrations 40 times greater than those of cytochrome *c* or NBT, to decrease the rates of cytochrome *c* or NBT reduction suggests that their reactions with O_2^- , if any, proceed with rate constants of $10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ or less. This lack of reaction is not unexpected from the structure of the compounds (Fig. 1), since neither amino acids nor imidazole compounds react with O_2^- in aqueous solution at significant rates [21].

Action on H_2O_2

H_2O_2 (final concentration 7.06 mM) and various concentrations of carnosine, anserine and homocarnosine were incubated at pH 7.4 for up to 30 min as described in the Materials and methods section, and the remaining H_2O_2 was measured either by a peroxidase-based method or by titration with $KMnO_4$ after acidification. No significant consumption of H_2O_2 was observed by either method. Since the peroxidase-based assay procedure can detect reaction of substances with H_2O_2 even with rate constants of less than $10 \text{ M}^{-1} \cdot \text{s}^{-1}$ [22], it is concluded that there is no significant reaction of carnosine, homocarnosine or anserine with H_2O_2 .

Scavenging of HOCl

HOCl is produced when Cl^- is oxidized by the enzyme myeloperoxidase [10]. One of the most important targets attacked by HOCl *in vivo* is α_1 -AP, the major circulating inhibitor of serine proteases such as elastase [10]. α_1 -AP is rapidly inactivated by HOCl, losing its ability to inhibit elastase. Many compounds can react with HOCl, but few do so sufficiently rapidly to protect α_1 -AP against inactivation [11,22–24]. A good test of whether a putative scavenger of HOCl is likely to scavenge *in vivo* is therefore

Table 1. Inactivation of α_1 -AP by HOCl: effect of scavengers

α_1 -AP (0.2 mg/ml), HOCl (60 μ M) and scavenger (if any) were incubated in a final volume of 1.0 ml in phosphate-buffered saline (PBS), pH 7.4, at 25 °C for 30 min [11]. Then 2 ml of PBS and 0.05 ml of porcine pancreatic elastase were added, followed by further incubation at 25 °C for 20 min to allow any α_1 -AP still active to inhibit elastase. (Any HOCl remaining is diluted out to the point at which it cannot affect elastase itself [11].) The remaining elastase activity was then measured by adding elastase substrate [11], resulting in an increase in A_{410} . Concentrations of scavengers added were those present in the first (1.0 ml) reaction mixture; unless otherwise indicated, scavengers and α_1 -AP were mixed together before adding HOCl. Control experiments showed that none of the substances tested themselves affected elastase activity or interfered with the ability of α_1 -AP to inhibit it. The higher the elastase activity measured, the greater the inhibition of α_1 -AP by HOCl. Thus a good scavenger of HOCl protects the α_1 -AP, so that its inhibitory activity is retained and measured elastase activities decline. Results are the means of two experiments that differed by < 13 %.

Addition to first reaction mixture	Elastase activity in final reaction mixture (A_{410}/s)	α_1 -AP activity (% inhibition of elastase)
Buffer only	1.38×10^{-2}	–
α_1 -AP	0	100
α_1 -AP + HOCl	1.24×10^{-2}	10
α_1 -AP + HOCl + 4.8 mM-carnosine	1.29×10^{-2}	7
α_1 -AP + HOCl + 7.2 mM-carnosine	1.10×10^{-2}	20
α_1 -AP + HOCl + 2.4 mM-anserine	1.32×10^{-2}	4
α_1 -AP + HOCl + 4.8 mM-anserine	9.7×10^{-3}	30
α_1 -AP + HOCl + 7.2 mM-anserine	9.0×10^{-3}	35
α_1 -AP + HOCl + 4.8 mM-homocarnosine	1.29×10^{-2}	7
α_1 -AP + HOCl + 7.2 mM-homocarnosine	1.28×10^{-2}	7
HOCl preincubated with 7.2 mM-homocarnosine for 20 min at 25 °C, then α_1 -AP added and incubation as above	1.32×10^{-2}	4
HOCl preincubated with 7.2 mM-carnosine, as above	0	100
HOCl preincubated with 7.2 mM-anserine, as above	0	100

to examine its ability, at the concentrations achieved *in vivo*, to protect α_1 -AP against inactivation by HOCl [11,22–24].

Table 1 (second line) shows that α_1 -AP inhibits the action of elastase; a concentration just sufficient to inhibit elastase completely was used. Incubation of α_1 -AP with HOCl led to loss of its elastase-inhibitor capacity (Table 1, line 3). Inclusion of carnosine or homocarnosine, at concentrations of up to 7.2 mM, in the reaction mixture did not protect the α_1 -AP. Anserine offered a slight protective effect [7.2 mM caused inhibition by $32 \pm 7\%$ in five experiments (means $\pm$ S.D)]. Hence these three compounds, even at 7.2 mM concentrations, cannot protect α_1 -AP against 60 μ M-HOCl. If the carnosine and anserine were preincubated with HOCl for 30 min before adding α_1 -AP, the α_1 -AP was completely protected. This suggests that carnosine and anserine do react with HOCl, but only slowly. By contrast, homocarnosine offered no protection even on preincubation (Table 1).

Scavenging of $\cdot$ OH and interference with iron-dependent production of $\cdot$ OH

A mixture of ascorbic acid, H_2O_2 and a suitable iron complex generates $\cdot$ OH at pH 7.4. This $\cdot$ OH may be measured by its ability to degrade the sugar deoxyribose into fragments that, on heating with TBA at low pH, generate a pink chromogen [12]. If iron is added to the reaction mixture as an $FeCl_3$ –EDTA complex, any $\cdot$ OH that escapes scavenging by the EDTA is equally accessible to deoxyribose and to any added scavenger of $\cdot$ OH [11]. Thus the ability of a scavenger to inhibit deoxyribose

degradation in this system depends only on its concentration relative to deoxyribose and on its second-order rate constant for reaction with $\cdot$ OH [12,25–27].

Fig. 2 shows the ability of carnosine, anserine and homocarnosine to inhibit deoxyribose degradation in this system. From the slopes of the competition plots, second-order rate constants can be calculated for their reactions with $\cdot$ OH. These are listed in Table 2, with some other data for comparison. It may be seen that all three compounds are good scavengers of $\cdot$ OH, as are histidine, histamine or imidazole. None of the compounds tested could themselves be degraded to produce TBA-reactive material (shown by controls in the absence of deoxyribose). Concentrations up to 8 mM (the maximum used in these studies; Fig. 2) produced little or no interference with the assay of deoxyribose degradation (tested by adding the compounds at the end of the incubation, just before addition of TBA and acid).

If iron is added to the deoxyribose assay system as $FeCl_3$ (not complexed with EDTA), some of the Fe^{3+} bind to deoxyribose [30], and deoxyribose degradation becomes site-specific, i.e. $\cdot$ OH is formed by Fe^{2+} bound to deoxyribose and immediately attacks this sugar. Most $\cdot$ OH scavengers fail to inhibit this deoxyribose degradation. Indeed, the only molecules that can prevent deoxyribose degradation under these conditions are those that themselves complex iron ions, as chelates poorly reactive in generating $\cdot$ OH, and so remove iron ions from the deoxyribose [25–27,30]. Thus the deoxyribose assay under these reaction conditions provides a simple test of the ability of compounds to bind iron ions in ways

Fig. 2. Inhibition of $\cdot\text{OH}$ -dependent deoxyribose (DR) degradation by carnosine, anserine and homocarnosine

Experiments were carried out as in [12]. A is the absorbance at the end of the experiment. The rate constants were determined from the slopes of the lines obtained in the presence of EDTA ($k = \text{slope} \times k_{\text{DR}} \times [\text{DR}] \times A^\circ$) where $k_{\text{DR}} = 3.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, $[\text{DR}]$ was 2.8 mM and A° is the absorbance obtained in the absence of scavenger. ●, Iron added to reaction mixture as FeCl_3 (final concentration 20 μM) ■, iron added to reaction mixture as FeCl_3 (20 μM)/EDTA (100 μM), pre-mixed before addition. (a) Carnosine, (b) homocarnosine, (c), anserine.

that depress the ability of these ions to participate in generation of $\cdot\text{OH}$ [27].

Fig. 2 shows that carnosine, anserine and homocarnosine had only slight effects on deoxyribose degradation in the presence of FeCl_3 . This suggests that their

Table 2. Rate constants for reactions with $\cdot\text{OH}$ as determined by the deoxyribose method

Results are the means of three or more determinations, that differed by no more than 10%.

Compound tested	Second-order rate constant ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Reference
Carnosine	4.0×10^9	This study
Homocarnosine	2.6×10^9	This study
Anserine	5.2×10^9	This study
L-Alanine	8.1×10^7	This study
L-Histidine	7.1×10^9	This study
Imidazole	4.8×10^9	This study
Histamine	5.0×10^9	This study
Mannitol	2.7×10^9	[28]
Glucose	1.0×10^9	[29]

ability to bind iron ions, in ways that diminish Fe-dependent $\cdot\text{OH}$ generation, is very limited.

Binding of Cu^{2+}

It has been suggested that carnosine may inhibit oxidative damage by binding Cu^{2+} [1]. Gutteridge [17] has developed an assay to detect Cu^{2+} capable of stimulating radical reactions, the 'phenanthroline assay'. If 10 μM - CuCl_2 was pre-mixed with 5 mM concentrations of anserine, homocarnosine or carnosine and incubated for 10 min at 25 $^\circ\text{C}$, the Cu^{2+} was still fully reactive in the phenanthroline assay. Control experiments showed that the compounds did not interfere with the assay of DNA degradation, nor did any complexes of these compounds with Cu^{2+} degrade DNA in the absence of phenanthroline.

Action on lipid peroxidation

Homocarnosine or β -alanine, tested at concentrations up to 20 mM, had no reproducible effect on the peroxidation of ox-brain phospholipid liposomes or rat liver microsomes at pH 7.4, as measured by a TBA test using HCl as the acid (see the Materials and methods section). Peroxidation was stimulated by adding FeCl_3 -ascorbate, Fe^{2+} , $\text{Fe}^{2+}/\text{Fe}^{3+}$, Fe^{2+} -ADP or Fe^{3+} -ADP/NADPH (the last in the case of microsomes only). However, both carnosine and anserine produced a concentration-dependent inhibitory effect in all these systems, in agreement with previous reports ([2], and references cited therein) Fig. 3 shows a representative result.

Unfortunately, control experiments revealed that some of this apparent inhibition was due to interference with the TBA assay itself rather than to a real inhibition of peroxidation. Table 3 shows one such control experiment, in which inhibitors of lipid peroxidation were present throughout the assay, or alternatively added at the end of the assay, immediately before addition of TBA and HCl. In the case of the chelator desferrioxamine, which inhibits Fe-dependent lipid peroxidation [31,32], its inhibitory effects under our reaction conditions were exerted during the assay itself and not during the TBA test; when desferrioxamine was added with the TBA reagents, it had little effect (Table 3, last column). Similar results (not shown) were obtained with the lipophilic chain-

Fig. 3. Apparent inhibition of lipid peroxidation by anserine (■), carnosine (○) and homocarnosine (●).

In this system, peroxidation of rat liver microsomes was induced by adding FeCl_3 and ascorbic acid; similar inhibitory effects were observed with liposomes, or if microsomal peroxidation was stimulated by adding Fe^{2+} , $\text{Fe}^{2+}/\text{Fe}^{3+}$ or Fe^{3+} -ADP/NADPH. Peroxidation was measured by the HCl-based TBA test: 1 % inhibition gave an A_{532} value of 1.02.

breaking antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). However, inhibitions by carnosine and anserine were still seen when they were added with the TBA reagents, although the inhibitions were somewhat less than when these substances were present at the start of the incubation.

It follows that carnosine and anserine appear to have some inhibitory effect on lipid peroxidation, but much of their apparent effect is due to interference with a form of the TBA test that has frequently been used in previous studies with these substances (reviewed in [2]). In order to investigate further the action of these substances, four different assays were used. Firstly, replacing the HCl in the TBA assay with trichloroacetic acid substantially

lessened the interference (Table 3). Carnosine and anserine, but not homocarnosine or β -alanine, showed weak inhibitory effects in this system. By contrast, histidine markedly stimulated peroxidation (Table 4); it was also stimulatory when an HCl-based TBA test was used (results not shown). Three completely different assays were also employed, one based on the accumulation of fluorescent chromophores in peroxidizing lipid systems [14,15,33,34], one based on diene conjugation [16] and the third based on measurement of O_2 uptake in an O_2 electrode [16]. A control experiment showed that carnosine, anserine or homocarnosine had no inhibitory effects on the measurement of fluorescent products, in that addition of them at the end of the experiment (before measurement of fluorescent products) had no effect. Homocarnosine or β -alanine had no action on peroxidation in the fluorescence assay, whereas carnosine and anserine were reproducibly inhibitory (Table 4 shows some results for microsomes; comparable results were obtained with liposomes). However, when peroxidation was measured by O_2 uptake, anserine and, to a lesser extent carnosine, stimulated O_2 uptake, as did histidine (but to a much greater extent). β -Alanine or homocarnosine had no reproducible effect, whereas desferrioxamine inhibited as expected. By contrast, in studies of diene conjugation, no clear-cut stimulatory or inhibitory effects of carnosine, anserine or homocarnosine could be demonstrated using liposomes or microsomes.

DISCUSSION

Carnosine, anserine and homocarnosine do not react with O_2^- or with H_2O_2 at significant rates, nor can they protect α_1 -AP against inactivation by HOCl. This is probably because they react too slowly with HOCl to compete with α_1 -AP. Another possibility is that the products of reaction of homocarnosine, anserine and carnosine with HOCl are themselves sufficiently oxidizing to inactivate α_1 -AP, in the same way that taurine reacts with HOCl to produce a secondary 'long-lived' oxidant that is capable of attacking α_1 -AP [10]. This is perhaps less likely for anserine and carnosine, since preincubation of the HOCl with them resulted in complete protection of

Table 3. Effects of anserine and carnosine on lipid peroxidation: interference with the TBA assay system

Peroxidation in rat-liver microsomes was studied. The TBA test was carried out using HCl as the acid, except where indicated, when trichloroacetic acid was used.

Antioxidant added... Point at which antioxidant added...		Inhibition of peroxidation (%)					
		12 mM- Carnosine		12 mM- Anserine		0.2 mM- Desferrioxamine	
Addition	Extent of peroxidation (<i>A</i> ₅₃₂)	Start of expt.	During	Start of expt.	During	Start of expt.	During
			TBA assay only		TBA assay only		TBA assay only
Fe ³⁺ /ascorbate	1.18 (100 %)	75	61	80	53	84	7
Fe ³⁺ /ascorbate (TCA as acid)	0.88 (100 %)	57	28	66	29	92	3
Fe ³⁺ –ADP/NADPH	1.22 (100 %)	79	54	81	40	80	0
Fe ²⁺	1.05 (100 %)	66	58	54	50	75	5
Fe ²⁺ –ADP	1.13 (100 %)	69	56	62	48	75	13

Table 4. Action of carnosine, homocarnosine, anserine and their components on microsomal lipid peroxidation

Reaction mixtures, in a total volume of 1 ml, contained 10 mM-KH₂PO₄/KOH buffer, pH 7.4, 100 μ M-FeCl₃, 100 μ M-ascorbate and 0.4 mg of microsomal protein. For the TBA test, their incubation was at 25 °C for 10 min before performing the test using trichloroacetic acid as the acid. Less than 15% of any effects observed were seen when additions were made with the TBA reagents. For O₂ uptake, experiments were performed in a Hansatech O₂ electrode and the initial rate of O₂ uptake after any lag period had finished are presented; results shown have been corrected for any rates of O₂ uptake observed when either microsomes or FeCl₃/ascorbate had been omitted from the reaction mixture. These rates were < 10% of rates of O₂ uptake in the complete reaction mixture. The concentrations stated below are final concentrations in the reaction mixture. RFI, relative fluorescence intensity; NT, not tested in this experiment.

Addition to reaction mixture	Extent of peroxidation		
	TBA/TCA assay* (A ₅₃₂)	Fluorescence assay* (RFI)	O ₂ uptake† (μ mol of O ₂ /h)
None	0.261	105	0.24 ± 0.03 (9)
10 mM-Carnosine	0.173	40	0.30 ± 0.04 (5)
10 mM-Anserine	0.145	38	0.39 ± 0.05 (4)
10 mM- β -Alanine	0.249	104	0.26 ± 0.02 (2)
10 mM-Homocarnosine	0.305	103	0.29 ± 0.04 (3)
1 mM-Desferrioxamine	0.016	NT	0.02 ± 0.004 (2)
10 mM-Histidine	0.777	NT	1.28 ± 0.18 (4)

* Results of a representative experiment are shown; figures presented are means of duplicate determinations that differed by no more than 10%.

† Results are means $\pm$ S.D.; *n* values in parentheses. Effects of anserine, desferrioxamine and histidine are significant (*P* < 0.001); effects of β -alanine, homocarnosine are not significant at this level.

α_1 -AP (Table 1). In either case, it may be concluded that carnosine, anserine and homocarnosine are unlikely to offer protection *in vivo* to important biological targets, such as α_1 -AP, by scavenging HOCl.

Carnosine, anserine and homocarnosine are good scavengers of $\cdot$ OH, somewhat more effective than such compounds as glucose and mannitol but comparable with histidine (Table 2). Since high concentrations of the first three compounds are present *in vivo* (reviewed in [1]), scavenging of $\cdot$ OH would be feasible. None of the first three compounds appears to bind iron ions in a way that interferes with formation of $\cdot$ OH radicals in the deoxy-ribose assay. They might be able to bind Cu²⁺ and prevent some Cu²⁺-dependent radical reactions [1], but any such binding is insufficiently strong to prevent reactivity of Cu²⁺ in the phenanthroline assay. It must be pointed out that there has been considerable debate about whether copper ion complexes capable of stimulating radical reactions actually exist *in vivo* in mammals [17,35,36].

Homocarnosine had no action upon lipid peroxidation using any of the assay systems. Previous studies of the ability of carnosine and anserine to inhibit peroxidation may have been flawed because many of them used an HCl-based TBA test (reviewed in [2]), with which these substances have been shown to interfere. When peroxidation was assayed by a trichloroacetic acid-based TBA test or by fluorescence, some inhibitory activity was still observed at high concentrations of carnosine and anserine. However, measurements of diene conjugation showed no reproducible effect and studies of O₂ uptake showed, if anything, a stimulation of lipid peroxidation (control experiments showed that this was not due to direct oxidation of these substrates by microsomes, or to stimulation of O₂ uptake by Fe²⁺/Fe³⁺/reductant mixtures in the absence of lipids). By contrast, desferri-

oxamine, BHA and BHT inhibited reproducibly in all assay systems, and histidine reproducibly stimulated peroxidation (Table 4 shows some data for the TBA test and O₂ uptake). The ability of histidine to accelerate Fe-dependent peroxidation has already been reported [37,38]. The carnosine and anserine were not contaminated with free histidine (see below).

How can these variable results be explained? If it is assumed that anserine and carnosine do not really inhibit peroxidation (O₂ uptake, diene conjugation studies), it can be argued that they could interfere with fluorescence measurements, e.g. by reacting with the necessary aldehydes [34] rather than by inhibiting the chain reaction of lipid peroxidation. The TBA also relies on aldehyde production and could suffer from a similar artefact. Thus we conclude that reports of carnosine and anserine as inhibitors of peroxidation cannot be rigorously confirmed.

Our data raise another interesting point. Carnosine and anserine weakly inhibit lipid peroxidation in some assays, interfere with the HCl-based TBA test and can react on preincubation with HOCl so as to protect α_1 -AP added subsequently (Table 1). Homocarnosine does none of these things, yet its structure differs from that of carnosine only by the presence of an additional -CH₂- group (Fig. 1). Boldyrev *et al.* [39] have recently commented that the apparent 'antioxidative effect' of homocarnosine in a different lipid system is much less than that of carnosine or anserine, in agreement with our data. Why should this be? The chemical identity of the homocarnosine, anserine and carnosine samples used was checked by h.p.l.c.; only single sharp peaks co-eluting with authentic standards were observed. After hydrolysis using 10 M-HCl at 100 °C for 24 h, high-voltage electrophoresis showed the presence of the expected hydrolysis products, and no others. The ab-

sorption spectra of the compounds were also consistent with their stated identity. One remote possibility is that the apparent ability of carnosine and anserine to inhibit peroxidation weakly in some assays is due to the presence of antioxidant impurities in the preparations that are not present in homocarnosine, but no additional peaks were detected by h.p.l.c. Suppose, however, that undetected antioxidant impurities were present: this would strengthen our argument that the antioxidant capacity of these compounds has been overrated. One can only suppose that some critical conformation of anserine and carnosine, not present in homocarnosine, is required for their antioxidant activity. It is notable that differences in the physiological effects of these compounds have been reported. Thus carnosine had striking effects on the blood pressure of cats, whereas homocarnosine had no action whatsoever [40].

It therefore seems that carnosine, anserine and homocarnosine are unable to scavenge O_2^- , H_2O_2 or $HOCl$ at rates that could offer antioxidant protection *in vivo*, nor can they bind Fe^{3+} in ways that affect radical reactions, nor are they good inhibitors of lipid peroxidation. It may be that they accumulate merely to act as intracellular buffers, presumably due to the imidazole ring. If so, they might be safer to accumulate than histidine, which seems to be a powerful pro-oxidant in Fe-dependent lipid peroxidation [37,38; Table 4] and in other [41] systems.

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