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Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain

(histidine/lipid peroxidation/8-hydroxydeoxyguanosine)

RON KOHEN, YORIHIRO YAMAMOTO, KENNETH C. CUNDY, AND BRUCE N. AMES*

Department of Biochemistry, University of California, Berkeley, CA 94720

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ABSTRACT **Carnosine, homocarnosine, and anserine are present in high concentrations in the muscle and brain of many animals and humans. However, their exact function is not clear. The antioxidant activity of these compounds has been examined by testing their peroxyl radical-trapping ability at physiological concentrations. Carnosine, homocarnosine, anserine, and other histidine derivatives all showed antioxidant activity. All of these compounds showing peroxyl radical-trapping activity were also electrochemically active as reducing agents in cyclic voltammetric measurements. Furthermore, carnosine inhibited the oxidative hydroxylation of deoxyguanosine induced by ascorbic acid and copper ions. Other roles of carnosine, such as chelation of metal ions, quenching of singlet oxygen, and binding of hydroperoxides, are also discussed. The data suggest a role for these histidine-related compounds as endogenous antioxidants in brain and muscle.**

One of the processes involved in the adaptation of organisms to live in an aerobic atmosphere was the development of mechanisms for defense against damage induced by oxygen and active oxygen species (1). Active oxygen has been suggested as a major cause of cancer, aging, and several diseases (1–6). These reactive compounds can react with DNA, RNA, lipids, and proteins (1–7).

Natural defense mechanisms vary from one species to another and within the tissues of the same species. Skeletal muscle and brain are two of the tissues that have the most active oxidative metabolism, yet the concentrations of the antioxidants vitamin E and vitamin C in these tissues are not particularly high (8, 9).

Carnosine (β-alanyl-L-histidine) was discovered at the beginning of the century in skeletal muscle (10). Since then, carnosine and related compounds anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) have been reported (11) to be present in the range of 1–20 mM in the skeletal muscles of many vertebrates. There are high levels of carnosine in human muscles (2–20 mM) (11), olfactory epithelium and bulbs (0.3 mM–5 mM) (12–15), and in other parts of the brain. Homocarnosine is present in cerebrospinal fluid and brain (2–50 μM) (12–17), and anserine is present in the brain (18).

Although it is accepted that carnosine and its analogues should play some physiological role in muscle and brain, no unified hypothesis exists that can satisfactorily explain their role (18). Carnosine has been postulated to act as a buffer to neutralize lactic acid produced in skeletal muscle that is undergoing anaerobic glycolysis (19). Carnosine and anserine have been shown to be efficient copper-chelating agents, and it has been suggested that they may play a role in copper metabolism *in vivo* (18). Carnosine has been shown to have a specific binding site in albumin, although no function for this has been postulated (18). Other possible roles for these compounds have been suggested: as putative neurotransmitters in the olfactory bulbs (20), as a physiological activator for myosin ATPase (21), and as regulators of other enzymes (22).

Hartman and coworkers (23–25) have shown that carnosine is an efficient singlet-oxygen scavenger, quenching singlet oxygen more effectively than histidine, and that carnosine, anserine, and histidine protect phage against γ-irradiation, which gives rise to oxidative DNA damage. Bondarenko and coworkers (26–28) have shown that homocarnosine protects against the convulsions caused by the exposure of animals to hyperbaric oxygen and that its concentration decreases in rabbits after they have been exposed to hyperoxia. Carnosine also protects rabbit hearts from reperfusion injury after ischemia (29).

We present additional evidence for the role of carnosine and its analogues as protectors against oxidative stress and suggest that it and related compounds may serve as natural antioxidants in skeletal muscle and brain.

MATERIALS AND METHODS

Materials were purchased from Sigma unless otherwise stated. A chloroform solution of soybean phosphatidylcholine (PtdCho) was purified on a silica gel column; antioxidant was removed by chloroform/methanol, 95:5 (vol/vol), and PtdCho was eluted with chloroform/methanol, 60:40 (vol/vol).

HPLC. A Waters Associates model 510 pump with a model U6K injector system was used. The effluent was monitored at 234 nm with a Kratos (Westwood, NJ) 773 spectrophotometer. Data were digitized by a Nelson-760 (Cupertino, CA) analytical interface and were processed by Nelson series 4000x Xtrachrom data system 7.1 on a Hewlett–Packard 9816 computer.

Assay for the Lipid Peroxyl Radical-Trapping Activity. The azo compounds 2,2′-azobis(2,4-dimethylvoleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane dihydrochloride) (AAPH) (Polyscience, Warrington, PA) were used as free-radical initiators in both homogeneous and liposome systems (30–32). The rate of peroxyl-radical formation from these initiators is constant at a given temperature and, once produced, can initiate free-radical chain oxidation of lipid.

The oxidation of linoleic acid (148 mM) in methanol/water, 90:10 (vol/vol), was initiated with AMVN (10 mM) at 37°C in a water bath (Gyrotory C-70) under air. The free-radical chain oxidation produces linoleic acid hydroperoxide quantitatively at the initial stage (31, 32). The hydroperoxide was determined by injection of 10 μl of the reaction solution onto a 25-cm octadecylsilane (ODS) LC-18 column (Supelco,

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Abbreviations: PtdCho, phosphatidylcholine; CV, cyclic voltammetry; 8-OHdGuo, 8-hydroxydeoxyguanosine; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AAPH, 2,2′-azobis(2-amidinopropane dihydrochloride); GABA, γ-aminobutyric acid.

*To whom reprint requests should be addressed.

Bellefonte, PA), with 90:10 methanol/water as eluent at a flow rate of 1 ml/min. The effluent was monitored at 234 nm, as described above.

The oxidation of soybean PtdCho (2.86 mM) as multilamellar liposomes in an aqueous dispersion was also carried out at 37°C under air, and the water-soluble radical initiator AAPH (10 mM) was used (30, 32). The reaction solution was prepared as follows: 5 ml of methanol was added to 4 ml of a solution of PtdCho in benzene (5 mg/ml), solvents were removed by evaporation on a water aspirator with a rotary evaporator (Buchi, Switzerland) to obtain a thin film of PtdCho, 4.5 ml of phosphate-buffered saline was added, and the PtdCho film was slowly peeled from the flask by shaking to obtain a white, milky liposome solution. To start the reaction, 1 ml of a solution of AAPH and the compound to be tested in phosphate-buffered saline was added to 1 ml of liposome solution. The oxidation of soybean PtdCho can be measured by the formation of PtdCho hydroperoxide (PtdCho-OOH) (30). The reaction was carried out at 37°C with 10 mM AAPH, 2.86 mM soybean PtdCho liposomes, and additives, and the pH was adjusted to 7.2–7.6. The PtdCho-OOH produced was analyzed by injection at various time intervals of 10 μl onto a HPLC system using a silica gel column (Accupac-short, Rainin, Woburn, MA) with 90:10 methanol/water as eluent at a flow rate of 1 ml/min.

Inhibition by Carnosine of the Formation of 8-Hydroxydeoxyguanosine (8-OHdGuo). Deoxyguanosine (1 mM) was incubated at 37°C for 45 min in the presence of copper sulfate (100 μM) and ascorbic acid (1 mM) with additions when specified. Aliquots of 100 μl were injected onto a 25-cm ODS column (particle size, 5 μm; Supelco LC-18) preceded by a guard column containing the same resin. The mobile phase was 50 mM phosphate buffer, pH 6.8/methanol, 90:10 (vol/vol), and the flow rate was 1 ml/min. Since 8-OHdGuo has been shown to be electrochemically active (33), a BAS-LC4B electrochemical detector was used for quantitation (potential of +0.8 V vs. Ag/AgCl, with a glassy-carbon working electrode). The 8-OHdGuo standard was synthesized (34).

Cyclic Voltammetry (CV). A BAS (West Lafayette, IN) model CV-58 CV apparatus was used with a glassy-carbon working electrode and a Ag/AgCl reference electrode, modified for a 250-μl cell volume. The measurements were carried out between 0 and +2.0 V or −1.5 and +2.0 V. When CV was performed at negative potentials, the sample was bubbled with high-purity nitrogen for 60 min to remove interfering oxygen. The experiments were carried out in water/phosphate-buffered saline, 1:1 (vol/vol), at a scan rate of 100 $mV \cdot s^{-1}$. The pH of all solutions tested was adjusted to 7.2–7.6. All the experiments were repeated at least three times; the differences were <5% ($P < 0.05$).

RESULTS

Antioxidant Activity in the AMVN System. When carnosine was introduced into the reaction mixture containing AMVN and linoleic acid at 37°C, a decrease in the rate of oxidation of linoleic acid to linoleic acid hydroperoxide was recorded. Fig. 1 shows the oxidation of linoleic acid in aqueous methanol in the presence and absence of carnosine. The rate of formation of linoleic acid hydroperoxide in the control experiment was 15 $\mu M \cdot min^{-1}$. Carnosine at concentrations of 1, 7.5, and 10 mM reduced the rate of the oxidation of the linoleic acid, with rates of 14, 6.5, and 4 $\mu M \cdot min^{-1}$, respectively. Homocarnosine (10 mM) also showed a decrease in the rate of oxidation of the linoleic acid, being 35% as effective as carnosine (data not shown).

Antioxidant Activity in the AAPH System. The AAPH system is more physiological because the initiator is water soluble. The rate of oxidation of PtdCho in the absence of antioxidant (control) was found to be 3.6 $\mu M \cdot min^{-1}$ (Fig. 2, Table 1). The rates of oxidation for carnosine concentrations of 1, 5, 10, and 20 mM were 2.8, 1.9, 1.7, and 0.9 $\mu M \cdot min^{-1}$, respectively. Homocarnosine showed an antioxidant activity in the AAPH system (56% protection) similar to that of carnosine (53% protection) (Fig. 2, Table 1). Anserine nitrate showed a slightly greater inhibition of the oxidation of PtdCho (60% protection). Both anserine nitrate and homocarnosine, like carnosine (Fig. 1), showed a concentration-dependent antioxidant activity (data not shown).

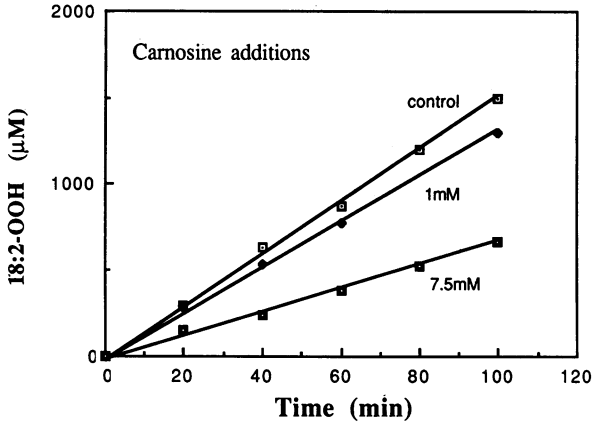

FIG. 1. The antioxidant activity of carnosine in the AMVN-induced oxidation system. The reaction was carried out at 37°C, and the reaction mixture contained AMVN (10 mM) as the peroxyl-radical initiator and linoleic acid (148 mM) as the substrate (control). Carnosine was added at 1 or 7.5 mM. 18:2-OOH is linoleic acid hydroperoxide.

Structure–Activity Relationships. To find out which part of the molecule is responsible for the antioxidant activity, a series of experiments were conducted, the results of which are summarized in Table 1. L-Alanyl-L-histidine, which differs from carnosine only in the form of the amino acid alanine (L-alanine vs. β-alanine), shows a less-pronounced antioxidant activity than carnosine (Table 1). The alanyl residues alone (L-alanine or β-alanine), the combination of both (L-alanyl-L-alanine or β-alanyl-L-alanine), and γ-aminobutyric acid (GABA) (present in homocarnosine) failed to show any antioxidant activity. The histidine moiety of the carnosine molecule was an efficient scavenger of the peroxyl radical (42% inhibition). Histamine, which lacks the carboxylic acid group of histidine, was not as efficient (28% inhibition).

Other derivatives of histidine were examined in an attempt to elucidate the mechanism of the antioxidant activity observed. The results obtained suggest that the imidazole ring

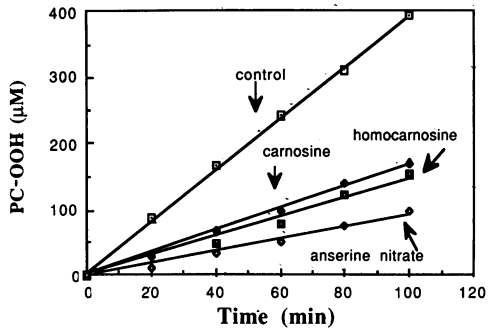

FIG. 2. The antioxidant activity of 10 mM carnosine, homocarnosine, and anserine in the AAPH-induced oxidation system. The reaction was carried out at 37°C, and the reaction mixture contained AAPH (10 mM) as the peroxyl-radical initiator and soybean PtdCho liposomes (2.86 mM) (control) with the three additives as indicated. PC-OOH is PtdCho hydroperoxide.

Table 1. Antioxidant activity of histidine-related compounds and controls in the AAPH-induced lipid oxidation system

Compound (10 mM)	Oxidation rate, $\mu M \cdot min^{-1}$	% inhibition
No addition	3.6	0
β-Alanyl-L-histidine*	1.7	53
L-Alanyl-L-histidine	2.2	39
L-Alanine	3.6	0
β-Alanine	3.6	0
L-Alanyl-L-alanine	3.6	0
β-Alanyl-L-alanine	3.6	0
Anserine nitrate	1.3	60
Homocarnosine	1.6	56
Histidine	2.1	42
Histamine	2.6	28
3-Methyl-L-histidine (τ)	1.6	56
1-Methyl-L-histidine (π)	2.2	36
GABA	3.6	0
Imidazole	2.2	39
1-Methylimidazole	3.4	6
4(5)-Methylimidazole	1.8	50
2-Methylimidazole	2.6	28
Sodium nitrate	3.6	0

*Carnosine.

is responsible for the antioxidant activity. Imidazole and 4(5)-methylimidazole were also active and showed a significant reduction of the rate of PtdCho oxidation. When one of the two nitrogens of the imidazole ring is methylated as in 1-methyl-L-histidine (π), 3-methyl-L-histidine (τ), or anserine, antioxidant properties were conserved, and 1-methylimidazole had little activity.

CV Measurements. In order to find out whether the various compounds could act as reducing agents, we recorded their cyclic voltammograms (Table 2). This activity should correlate with their antioxidant activity. Carnosine gave an anodic peak at a potential of +0.75 V and a second peak at +1.28 V, as shown in Fig. 3 and Table 2. Anserine nitrate was oxidized at a lower potential (E = +0.52 V),

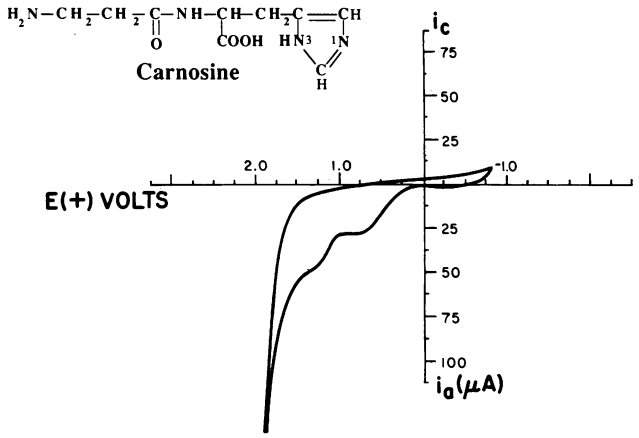

FIG. 3. Cyclic voltammogram of carnosine (20 mM). The carnosine was dissolved in water and diluted in phosphate-buffered saline (1:1), and the pH was adjusted to 7.4. CV was recorded with a glassy-carbon working electrode and Ag/AgCl as the reference electrode. The sample volume was 250 μl, and the sample was degassed extensively to remove oxygen before the measurement. The scan rate was 100 $mV \cdot s^{-1}$. i_a, Anodic current; i_c, cathodic current.

homocarnosine and histidine were oxidized at E = 1.2 V, while sodium nitrate, alanine, and GABA showed no response. The addition of a nonelectroactive residue, such as alanine or GABA, to the histidine or substituted histidine residue resulted in a reduction in the observed oxidation potential of the dipeptide (Table 2). Similar results were obtained for tyrosine and L-alanyl-L-tyrosine (Table 2).

Oxidation of Deoxyguanosine *in Vitro*. Carnosine prevented oxidative damage of deoxyguanosine induced by ascorbic acid and copper ions. Incubation of the reaction mixture resulted in the production of several deoxyguanosine adducts (Fig. 4), including 8-OHdGuo (elution of the 8-OHdGuo peak was found to be at 28 min by injection of a standard of 8-OHdGuo). When carnosine (1 mM) was added to the reaction mixture, no adducts were detected (Fig. 4*c*). Carno-

Table 2. Electrochemical response and antioxidant activity of various compounds

Compound (20 mM)	E, V	Electroactivity response	Antioxidant (10 mM) activity
β-Alanyl-L-histidine*	0.75, 1.28	++,+	++
Homocarnosine	1.23	+	++
Anserine nitrate	0.52	+	+++
L-Histidine	1.25	+	+
Sodium nitrate	–	–	–
GABA	–	–	–
L-Alanine	–	–	–
β-Alanine	–	–	–
L-Alanyl-L-alanine	–	–	–
β-Alanyl-L-alanine	–	–	–
1-Methyl-L-histidine (π)	1.30	+	+
3-Methyl-L-histidine (τ)	1.65	+	++
Imidazole	1.25	+++	+
1-Methylimidazole	–	–	–
4(5)-Methylimidazole	1.03	+	++
2-Methylimidazole	1.25	+	+
L-Tyrosine†	1.25	+	++
L-Alanyl-L-tyrosine†	0.90, 1.25	++,+	+++

The CV was recorded as described. The pH of the reaction mixtures was adjusted to 7.2–7.6. The electroactivity (magnitude of the peak anodic current) was marked as: +++ (strong response), ++ (moderate response), + (weak response), or – (no response). The antioxidant activity was measured using the AAPH-induced oxidation assay and marked as +++ (60% inhibition), ++ (45–60% inhibition), or + (<45% inhibition in the rate of oxidation of the control).

*Carnosine.

†The concentration was 0.5 mM.

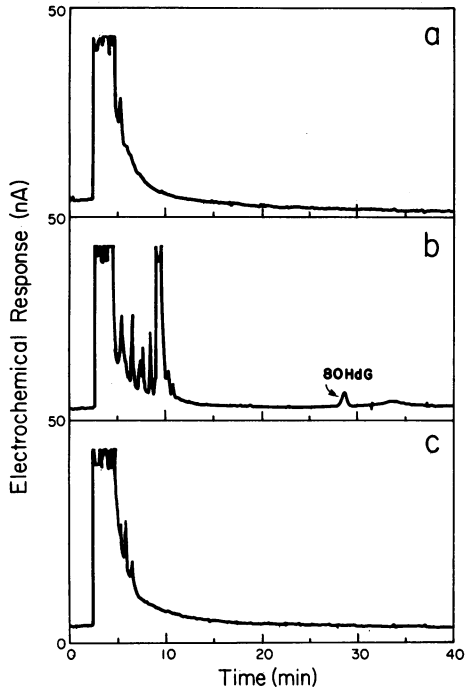

FIG. 4. An HPLC chromatogram of deoxyguanosine (1 mM) treated with ascorbic acid (1 mM) and copper (100 μM) at 37°C. (*a*) Zero time. (*b*) Forty-five-minute incubation. (*c*) Forty-five-minute incubation in the presence of carnosine (1 mM). The HPLC conditions were as described, and the injection volume was 100 μl. 8OHdG, 8-OHdGuo.

sine at a lower concentration (0.5 or 0.25 mM) also prevented the production of the 8-OHdGuo and partially prevented the production of the other unknown peaks in the chromatogram (Fig. 4*b*). Similar experiments with ferrous ions showed a different pattern of oxidation products, including a peak that corresponds to 8-OHdGuo. The formation of these products was also inhibited by carnosine (data not shown).

DISCUSSION

Antioxidant Role of Histidine-Related Compounds. The broad definition of a biological antioxidant includes compounds that can prevent oxidative damage to lipids, proteins, DNA, and other essential macromolecules. Most antioxidants show some specificity in that they provide only one general type of protection [i.e., blocking free-radical initiation; removing oxidants from the biological targets; reacting with the reactive species, thus sparing the biological target; transforming a reactive species to a nonreactive species; stabilizing membranes; and acting indirectly by removal of mediators that can catalyze free-radical damage (1, 2)]. Lipid-soluble antioxidants, for example, cannot give efficient protection in an aqueous environment. Various protective enzymes such as superoxide dismutase or catalase can only react with their substrates. A compound that shows several antioxidant properties should be of greater importance in preventing biological damage induced by reactive metabolites. Our experiments in the AMVN- and AAPH-induced lipid-oxidation systems and in the deoxyguanosine oxidation system indicate that carnosine and its analogues may possess several of these antioxidant properties and may play an important role in the defense against damage induced by oxidative stress *in vivo*.

Mechanism of Action. The antioxidant activity shown by histidine and its derivatives is due to the imidazole moiety of the molecule. Imidazole alone decreased the rate of oxidation of PtdCho liposomes by 39% (Table 1). 1-Methylimidazole, which lacks the proton on the nitrogen of the imidazole ring, showed little antioxidant activity, suggesting that the proton on the ring nitrogen is required for activity, although 1-methyl-L-histidine, 3-methyl-L-histidine, and anserine, which also lack this proton, were quite active. Neither the amino acid β-alanine nor the dipeptides (β-alanyl-L-alanine or L-alanyl-L-alanine) showed antioxidant activity (Table 1). These results exclude the possibility that the hydrogen next to the nitrogen in the amide bond can be donated. We conclude that the hydrogen on the ring nitrogen and that on the methylene carbon next to the imidazole ring are likely donors. By contrast, the enzymatic oxidation products of the imidazole ring are the 2- and 5-imidazolones. Imidazolones themselves have antioxidant activity (35).

Changes in the pH of the reaction mixture influenced the ability of histidine-related compounds to scavenge peroxyl radical; for example, a decrease in the pH also causes a decrease in the antioxidant activity (data not shown). During enhanced anaerobic glycolysis there is a production of lactic acid that would reduce the pH in the muscle. However, carnosine has a high buffering capacity (19), which helps to keep the pH around physiological values so that carnosine also will be able to cope with the oxidative stress.

Carnosine has a characteristic cyclic voltammogram (Fig. 3). Anserine and homocarnosine also give anodic CV responses, and the resulting anodic current indicates that these compounds are indeed reducing agents and, therefore, may act as antioxidants (Table 2). While carnosine and homocarnosine showed the same antioxidant ability, they differed in their oxidation potentials as shown by CV. 3-Methyl-L-histidine was oxidized at a relatively high potential (Table 2) but shows strong antioxidant activity. The alanine group contributes to the reduction in oxidation potential of these compounds, concomitant with the corresponding increase in antioxidant activity. β-Alanyl-L-histidine (carnosine) is oxidized at a lower potential than histidine, and the antioxidant activity observed was also greater. Similar results were obtained when tyrosine and L-alanyl-L-tyrosine were compared (Table 2). Thus, the ability of compounds to donate an electron as measured by the CV is generally related to their antioxidant activity in donating a hydrogen atom to the peroxyl radicals. However, this relationship has some complexities that need to be clarified.

Carnosine as a Chelating Agent. One of the mechanisms by which antioxidants can protect their biological targets from oxidative stress is the chelation of transition metals such as copper and iron, preventing them from participating in the deleterious Fenton reaction with peroxides. Carnosine and anserine have been shown to be very efficient copper-chelating agents (18). The ability of carnosine to inhibit the oxidation of deoxyguanosine induced by ascorbic acid plus copper ions (Fig. 4) may be due to the chelation of the copper by the carnosine in a form in which the copper is unreactive.

Human skeletal muscle contains one-third of the total copper in the body (20–47 μmol/kg) (18), so the presence of carnosine in this tissue at high concentrations could indicate its physiological role as a chelator of copper ions. Copper concentrations in the olfactory bulbs were found to be 50 μM, while carnosine and anserine concentrations are in the millimolar range, suggesting the possibility of the chelation of copper by these molecules. The protective effect of carnosine observed in the ascorbate–copper system could be explained in part also by other properties of the carnosine molecule, such as scavenging of free-radical intermediates and the binding of hydrogen peroxide (36).

The ability of carnosine and its analogues to bind iron (ferrous or ferric) has not yet been studied, but the binding of iron by the histidine residue itself is well documented (37) and could indicate that carnosine may also be an efficient

chelator of iron. The binding affinity of carnosine for other heavy metals such as cobalt has been reported (18).

Occurrence and Evolutionary Aspects of Carnosine. Carnosine, anserine, or other histidine derivatives are present in muscle tissues of most vertebrate species, except in the muscle of certain fishes, where free histidine can be detected at high levels (38, 39). Carnosine concentrations are also low in reptilia, except in the skin of some species (2 mM), and are low in mice (0.6 mM) (38, 39). A higher concentration can be found in rat (2 mM), and a much higher concentration can be found in larger animals such as horse (20 mM) and ox (13 mM) (39, 40). Levels in humans are quite high (up to to 20 mM). A significant elevation of carnosine in sprinters and rowers has been reported (40). One can interpret this as a possible physiological mechanism to help cope with the increased oxidative stress. Anserine is absent from human muscle (39), whereas it is present in high concentration in other animal muscles—e.g., 2.1 mM in cat, 43 mM in chicken, and 17 mM in rabbit leg muscle (38).

Carnosine and the Brain. One of the most likely places for oxidative stress is the human brain. The brain uses about 20% of the oxygen consumed (42), has the highest rate of oxygen consumption of any organ, and contains lipids with a high content of easily oxidized unsaturated fatty acids. Human cerebrospinal fluid contains the antioxidants ascorbic acid (about 100 μM) (8), urate (about 18 μM) (5), and homocarnosine (up to 50 μM) (12, 15–17). In brain tissue, carnosine, homocarnosine, and anserine were reported to be in the 0.3 mM to 5 mM range and together dominate the nonprotein nitrogenous compound pool of human brain (11–17). The distribution of these compounds in the brain is not homogenous, and the highest concentration is located in the olfactory bulbs (2–5 mM), whereas the concentrations in other regions of the neuronal system are lower. The different ways in which carnosine can act against oxidative stress and its high concentration in several parts of the brain could indicate its importance. In addition, an enzyme is present in the olfactory bulbs and in other parts of the brain that can synthesize carnosine (41), whereas ascorbic acid and vitamin E levels are completely dependent on diet.

Carnosine, homocarnosine, and anserine may be biologically significant antioxidants; they scavenge peroxyl radicals, are efficient chelating agents for copper and other transition metals, scavenge singlet oxygen (23, 24), and are present in high concentrations in skeletal muscle and brain—the two tissues with the most active oxidative metabolism.

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