Carnosine (β-alanyl-L-histidine) was discovered in 1900 as an abundant non-protein nitrogen-containing compound of meat. The dipeptide is not only found in skeletal muscle, but also in other excitable tissues. Most animals, except humans, also possess a methylated variant of carnosine, either anserine or ophidine/balenine, collectively called the histidine-containing dipeptides. This review aims to decipher the physiological roles of carnosine, based on its biochemical properties. The latter include pH-buffering, metal-ion chelation, and antioxidant capacity as well as the capacity to protect against formation of advanced glycation and lipoxidation end-products. For these reasons, the therapeutic potential of carnosine supplementation has been tested in numerous diseases in which ischemic or oxidative stress are involved. For several pathologies, such as diabetes and its complications, ocular disease, aging, and neurological disorders, promising preclinical and clinical results have been obtained. Also the pathophysiological relevance of serum carnosinase, the enzyme actively degrading carnosine into L-histidine and β-alanine, is discussed. The carnosine system has evolved as a pluripotent solution to a number of homeostatic challenges. L-Histidine, and more specifically its imidazole moiety, appears to be the prime bioactive component, whereas β-alanine is mainly regulating the synthesis of the dipeptide. This paper summarizes a century of scientific exploration on the (patho)physiological role of carnosine and related compounds. However, far more experiments in the fields of physiology and related disciplines (biology, pharmacology, genetics, molecular biology, etc.) are required to gain a full understanding of the function and applications of this intriguing molecule.
molecule (β-alanyl-Nτau-methyl-histidine), and preference is given to the name ophidine (348). Carnosine, anserine, and ophidine will be collectively called the histidine-containing dipeptides (HCD) in this paper.

II. DISTRIBUTION OF CARNOSINE AND ITS ANALOGS

A. Distribution of HCD in Animal Species

The distribution of HCD in various animal species has been the subject of study for nearly 100 years. Almost all studies have been conducted on skeletal muscle tissue. Therefore, we will mainly limit our overview of the literature to skeletal muscle, and discuss below the distribution of HCD in mammals and other vertebrates.

1. HCD in skeletal muscles of mammals

Based on a large number of comparative studies (64, 80, 91, 164, 405) and studies on individual species (20, 21, 86, 120, 193, 261, 309, 337) that have been conducted on the presence and distribution of carnosine, anserine, and ophidine in mammalian muscles, we have composed Figure 3. Data were available on 39 different species from only 9 (out of 21) mammalian orders, meaning that many orders of mammals, e.g., the Chiroptera (bats etc.), Insectivora, Proboscidea (elephants etc.), still remain to be studied.

Still, several conclusions can be drawn from Figure 3.
There is a large range in concentrations in total HCD content between different mammals, with some of the highest values reported in the wallaby and blue whale [50–60 mmol/kg wet weight (ww) or 12–14 g/kg ww, or more than 1% of the muscle’s ww] and some of the lowest reported in mice and opossum (1–3 mmol/kg ww or ~0.04% of the muscle’s ww).

As a rule, almost all mammals have both carnosine along with just one of the methylated carnosine analogs (either anserine or ophidine). The only mammalian species known not to have any of the two methylated carnosine analogs is in fact the Homo sapiens. Another rare exception is that some animals express all three HCD, such as the pig and the buffalo, but in these cases the methylated forms are in far minority compared with carnosine.

In most cases, the methylated form is more abundant than the nonmethylated carnosine itself. Entire mammalian orders follow this pattern, such as the Cetacea (marine mammals), Rodentia, and Marsupalia. In general, there is a good similarity in the HCD expression pattern between the different species within one family and the different families within one order.

Of the two methylated forms, anserine is more frequently observed than ophidine. Whereas anserine is found in nearly all mammalian orders, ophidine is only found in significant amounts in marine mammals (Cetacea) and in smaller amounts in some hoofed animals: the even-toed ungulates (Artiodactyla) and odd-toed ungulates (Perissodactyla). These three orders all belong to the same super-order of mammals, the Laurasiatheria.

2. HCD in muscles of other animals

In nature, HCD are probably only present in animals. At least nobody has ever detected them in plants, fungi, or other eukaryotes, even though it must be said that it is not clear how good the techniques were and how thorough the search was for the presence of known or possibly unknown HCD in non-animal eukaryotes. Within the animal kingdom, HCD have only been detected in vertebrates and not in invertebrates, except from the reported presence of high concentrations of anserine and carnosine in prawn, but this has not been confirmed in a peer-reviewed paper (202).

The distribution of HCD in fish species are shown in Figure 4, a compilation of data based on References 2, 4, 20, 21, 61,
It becomes clear that some of the fish species (such as herring, sardine, carp, sole, etc.) contain very little, if any, HCD. However, several of these fishes contain high or very high concentrations of free L-histidine instead. This would suggest that the role of HCD is replaced by L-histidine and that this therefore mainly relates to the imidazole ring of L-histidine (e.g., the pH buffering function). Of all the examined fishes, it seems that they nearly all express only one type of HCD, either carnosine or anserine.

The HCD concentrations found in muscles of amphibians, birds, and reptiles are shown in FIGURE 5, a compilation based on References 2, 4, 21, 64, 80, 91, 201. All the investigated species in these classes consistently express at least one type of HCD: the amphibians carnosine, the birds anserine (sometimes along with carnosine), and the reptiles primarily ophidine (sometimes along with carnosine).

When considering the occurrence of the three HCD in all investigated vertebrate species (FIGURES 3–5), ophidine is almost exclusively seen in two unrelated lineages, i.e., in some snake families (order of Squamata) and some specific marine mammalian families (order of Cetacean). This most likely signifies that the enzymes for ophidine and anserine synthesis have evolved at least two times in evolution independently of each other, a phenomenon which is called convergent evolution (113).

B. Distribution of HCD in Tissues

In mammals, only two tissues have carnosine concentrations as high as in the millimolar range, i.e., skeletal muscle and the olfactory bulb. As only the former represents a considerable percentage of the total body mass, it can be calculated that over 99% of the carnosine present in an organism is found in the skeletal muscle tissue. Carnosine is measurable in other brain regions and other tissues and body fluids as well, but in concentrations 10- to 1,000-fold lower than in muscle (see FIGURE 6). A detailed discussion of the distribution of carnosine in the brain is given in section V.

There is only fragmentary and sometimes conflicting literature on the distribution of HCD in non-muscle tissues. There are reports of relatively high concentrations of HCD in the skin of reptiles (snake and skink) and frogs (91). Interestingly, the same HCD type that was present in the
muscle was present in skin (e.g., cobra snake has only ophidine as HCD in muscle and has only ophidine in skin tissue) (91). Some authors have reported the absence of HCD from liver (95, 96), whereas Purchas and Busboom (307) have reported very high bovine liver carnosine concentrations in the low end of the concentration range found in skeletal muscles (~4 mmol/kg). Others found trace amounts of carnosine in tissues such as the bovine lens (77). Part of the contradiction in the literature may have arisen from the fact that 1) carnosine concentrations in various tissues are at the limit of detection, 2) there are significant interspecies differences, and/or 3) there is a large difference in the accuracy of HCD quantification between the different analytic methodologies.

A recent profiling of HCD in rat tissues by the high-precision method of liquid chromatography/electrospray ionization tandem mass spectroscopy (ESI-MS)(13), revealed that carnosine was detectable in the millimolar range in skeletal muscle (2–6 mmol/kg wet tissue), in the micromolar range in brain, cerebellum, and myocardium (25–70 µmol/kg wet tissue) and nondetectable in kidney, liver, lung, and plasma. A similar profiling of carnosine content in human tissues is currently lacking in the literature. The only frequently measured human tissue carnosine concentration is that of skeletal muscle, because of the high concentration and the relative ease to sample muscle tissue invasively (biopsy; Ref. 243) or noninvasively (magnetic resonance spectroscopy; Ref. 286).

Mammalian cardiac muscle has only a relatively low concentration of carnosine (~0.1 mmol/kg wet tissue). How-
ever, the total content of carnosine derivatives is high and in the order of 2–10 mmol/kg wet tissue, as the concentrations of N-acetylated carnosine, anserine, and homocarnosine are reportedly present in the millimolar range in cardiac tissue (279, 280).

III. CHEMICAL AND BIOCHEMICAL PROPERTIES OF CARNOSINE

A. Acid-Base Properties and Buffering Activity

Carnosine is a very water-soluble compound (1 g in 3.1 ml of water at 25°C), characterized by three ionizable groups: the carboxylic group (pK_a 2.76), the amino group of the β-alanine residue (pK_a 9.32), and the nitrogens of the imidazole ring (pK_a 6.72) (384). Based on the pK_a values, at physiological pH, carnosine is mainly present in the zwitterionic form with both the carboxyl and amino group of β-alanine in their ionized states. The chemistry of the imidazole ring in carnosine is quite interesting and deserves some detailed explanation since it regulates the buffering activity of carnosine. By using Raman spectroscopy, Torreggiani et al. (371) reported that at pH 7 the bands caused by the positively charged and neutral forms of the imidazole ring are both visible, which indicates that at this pH value, both forms are present. The two tautomeric forms of carnosine’s imidazole ring in its neutral form are the Nτ protonated (tautomer I) and the Nπ protonated (tautomer II) (FIGURE 7). Raman studies (371) indicate that both tautomers exist at neutral pH, thus indicating an equilibrium between the two tautomeric forms. Furthermore, at pH 7 and 9, the tautomer I is the most predominant species (75%), in accordance the tautomer II being energetically less stable than the tautomer I (23). The tautomeric equilibrium is affected by metal chelation. Binding of a Cu(II) ion and dimer complex formation (vide infra) makes the tautomer II dominate since Nτ is involved in the metal chelation (370).

The nitrogen atoms of the imidazole ring of carnosine regulate the buffering activity of the dipeptide. It is suggested that because the pK_a values of carnosine and anserine are very close to 7.0, their proton sequestering capacity is high (322). The contribution of carnosine to the buffering capacity of skeletal and cardiac muscle will be discussed in more detail in section V.

B. Metal Ion Chelating Activity

Several analytical studies have been reported on the ability of carnosine to form complexes with metals of the first transition metal series such as Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Zn^{2+}. Copper and zinc complexes have been the most investigated ones, due to their biological relevance and are described below.

1. Copper-carnosine complex

Several models have been proposed for the structure of the Cu^{2+}-carnosine complex in aqueous solution since 1955. Dobbie and Kermack (108) first proposed the structure of Cu^{2+}-carnosine complex in which Cu^{2+} is bound to the imidazole, amino, and peptide nitrogen atoms, forming two six-membered chelate rings (FIGURE 8). The model was then confirmed by other authors who agreed on a monomeric form where Cu(II) is initially bound to the Nτ atom of imidazole and that, as the pH is increased, the amino and peptide nitrogen atoms deprotonate and bind to the metal (44).

Later, Freeman and Szymanski (44) have determined, by X-ray crystallography, the structure of the complex in the solid state which was not a monomer but a dimer in which the amino group, peptide nitrogen atom, and one carboxyl oxygen atom of one peptide molecule, and the Nτ atom of the imidazole ring of the second peptide molecule are bonded to Cu^{2+}. It was then proposed that the dimeric form exists in equilibrium with the monomer in solution and that the equilibrium is shifted towards the dimer at neutral pH. Later, by using different analytical techniques including ESI-MS (256) and Raman and IR spectroscopy (370–372), it has been found that, at neutral and basic pH and when Cu(II) and carnosine are mixed in a 1:1 molar ratio, the most relevant species formed is the dimer [CuH_2L_2]^0 which exists in equilibrium with the monomeric species. In the dimeric form, the ligand can scavenge the metal through the carboxylate, amide, and amino donor atoms. In the imidazole rings (tautomer II), the Nτ nitrogen atoms bind the copper(II) ions. The monomeric complexes [CuHL]^2+ and [CuL]^1+ are present at a somewhat lower pH. Consistently, the four chelation sites bound the Cu(II) ions and the complexes of Cu(II) and carnosine presumably have a square-planar coordination sphere (371).

Most of the studies on the characterization of Cu^{2+}-carnosine complexes have been carried out in aqueous solutions, but the existence of the dimer in living organisms was considered improbable due to the low copper concentration.
and the presence of competing ligands that would shield monomeric species with mixed ligands (75). Such consideration was confirmed by using localized $^1$H-NMR spectroscopy of human calf muscle. Schröder et al. (329) demonstrated that the dimer complex is insignificant in vivo, whereas the presence of a monomeric species was confirmed, although in a low amount. Finally, the paper by Velez et al. (382) should be carefully considered, because they have directly compared the metal ion binding properties of carnosine and L-histidine. Interestingly, carnosine, at pH 7.84 ± 0.18, was found to possess a much lower binding constant with Cu(II) ions (1.1 M$^{-1}$) compared with that of L-histidine (71 M$^{-1}$), as determined by Benesi-Hildebrand method.

2. Zinc-carnosine complex

There has been a recent research interest in the Zn(II)-carnosine complex because of its interesting and important pharmacological application (250). In fact, a zinc-carnosine complex (Polaprezinc or Z-103) was shown to protect gastric mucosa from experimental ulcerations in vivo (172, 218), and to be effective against Helicobacter pylori-associated gastritis (157, 195). Polaprezinc is a zinc complex that is dissolvable in acid and appears to have the characteristic to protect against mucosal lesions. It has a stable presence in the stomach and can adhere to ulcerous sites in a much better way than either zinc or carnosine alone (141). It is only possible to perform solution studies on the Zn(II)/carnosine system in a narrow pH range (129). At a pH of 6, there is an appreciable complex formation, but at higher pH (above 7.5) precipitation will take place even when the ligand is in excess (129).

The zinc(II)-carnosine complex was investigated at different pH values and metal/ligand ratios by Raman and IR spectroscopy by Torreggiani et al. (370). The dimer is the most prevalent species at neutral pH, especially if the ligand/metal ratio is 4, along with the charged monomeric complex, [ZnHL]$^{2+}$, in which the N-imidazole nitrogen contributes to the coordination. In more acidic conditions, the latter is also formed with monomeric complex, [ZnL]$^{+}$ as the most predominant. The existence at neutral pH of a dimeric form was confirmed by ESI-MS experiments (256).

In conclusion, most of the studies on carnosine as a chelator of Cu$^{2+}$ and Zn$^{2+}$ have been carried out in vitro and using conditions very far from the physiological ones. Hence, more studies are required not only to understand whether carnosine is an effective physiological chelator of metal ions but also to clarify the biological roles of such mechanisms.

C. Antioxidant Activity

The antioxidant activity of carnosine was initially studied by Boldyrev et al. since the 1980s (65–67). Later on, several studies were reported, showing a direct and indirect antioxidant activity of carnosine, as demonstrated in several in vitro studies (see below). The antioxidant activity of carnosine is mediated by different mechanisms involving metal ion chelation, and scavenging reactive oxygen species (ROS) and peroxyl radicals. The metal ion chelating properties of HCD have been discussed above and below the radical scavenging effect of HCD towards ROS and peroxyl radical is described.

1. Reaction of HCD with ROS, peroxynitrite, and hypochlorous acid

At physiological concentrations, carnosine was found to directly react with superoxide anion like superoxide dismu-
Carnosine as a scavenger of ·OH radicals. [Based on Tamba and Torreggiani (358).]

In the presence of copper, the fast oxidation of carnosine by ·OH still takes place (at 6.3 the formation rate constant is 7.9 × 10^9 M^{-1}·s^{-1})(369) and yields adducts of ·OH radicals at the imidazole ring (359). Thus the imidazole group is also the preferred site for ·OH binding in the presence of copper.

Imidazole compounds are known to react with singlet oxygen forming endoperoxide products. Accordingly, carnosine was reported to react with singlet oxygen two- to fourfold faster than free L-histidine and approximately twofold faster than the NH2-terminal L-histidine dipeptides tested (167). The rate constants of 1O2 quenching (Kq) by histidine containing peptides towards singlet oxygen was then determined by monitoring the 1O2-phosphorescence (1270 nm). The Kq values of carnosine and anserine were shown to be similar [(3 ± 1) × 10^7 M^{-1}·s^{-1}] and slightly higher in respect to that of imidazole [Kq = (2 ± 1) × 10^7 M^{-1}·s^{-1}]. The nonaromatic amino acids taurine and β-alanine showed very low quenching activities (Kq < 3 × 10^3 M^{-1}·s^{-1}) (124).

The ability of carnosine and related peptides to act as protective agents by counteracting the peroxynitrite-dependent reaction has been studied by Fontana et al. (133). The results showed that carnosine, at concentrations similar to those found in human tissues, efficiently protects tyrosine against nitration, α1-antiproteinase against inactivation and human low-density lipoprotein against modification by peroxynitrite. A similar protective effect was shown for anserine, and the scavenging activity of carnosine and anserine was ascribable to the imidazole moieties of the molecules, since L-histidine but not β-alanine is an effective inhibitor of peroxynitrite-induced tyrosine nitration (133).

Carnosine is also a highly effective protective agent against hypochlorite (HOCI). Hipkiss et al. (184) reported the ability of carnosine to dose-dependently inhibit protein cross-linking and high-molecular-weight oligomers induced by hypochlorite. The mechanism of reactions has been later studied by Pattison et al. (290) who found that carnosine, as well as other compounds containing imidazole and free amine sites, rapidly form imidazole chloramines. In the case of carnosine, a selective intramolecular chlorine transfer reaction occurs, leading a long-lived chloramine which, in contrast to chloramine formed by amino compounds, is inefficient at inducing further chlorination, thus limiting HOCI-mediated oxidation in vivo. The mechanism for the peculiar intramolecular Cl shift in carnosine from the imidazole ring (the kinetically favored site) to the terminal amine has been more recently studied by Karton et al. (210) who found that the intramolecular Cl shift in carnosine occurs due to a unique structural relationship between three adjacent functional groups, namely, the imidazole ring, the carboxylic group, and the β-aryl primary amine functions.

2. Reaction with peroxyl radicals

Kohen et al. (219) reported the ability of carnosine and its analogs such as anserine and homocarnosine to scavenge peroxyl radicals. They further demonstrated that the L-histidine moiety is required for this activity. The antioxidant and in particular the radical scavenging activity of carnosine was then confirmed by several other in vitro studies which also reported a reducing ability of carnosine that
Fe(III) to MbFe(II)O2, clearly indicating that the radical scavenging activity of carnosine was then reevaluated by Decker et al. (100) using a purified carnosine. They confirmed that purified carnosine was capable of scavenging peroxyl radicals at a millimolar concentration range, with a potency similar to that shown by L-histidine, while β-alanine was ineffective, thus suggesting that the radical scavenging activity of carnosine is largely due to the L-histidine residue. Purified carnosine was then found not able to reduce Mb-Fe(III) to MbFe(II)O2, clearly indicating that the radical scavenging activity of carnosine is not due to reducing properties. The fact that carnosine does not act as an electron transfer agent is also confirmed by the inability of carnosine to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (at least up to 1 mM), in contrast to the well-known reducing agents ascorbic acid or glutathione, that have this effect in the micromolar concentration range (14).

3. Overall antioxidant activity: in vitro models

The antioxidant activity of carnosine and related peptides has been demonstrated in several in vitro models, based on metal ion-dependent and -independent inducers of oxidative damage (metal ions/H2O2, radical inducers), with proteins, nucleic acids, and lipids as substrates, and based on a variety of methods for assessing the antioxidant capacity. When metal ions were used to generate free radicals, carnosine was observed to have a greater antioxidant activity when oxidation was promoted by copper than by iron (100). This is due to the ability of carnosine to chelate and deactivate copper ions while it can only chelate iron in a manner that it does not reduce its pro-oxidant activity (99). Overall, the efficacy of carnosine was usually found quite similar to that of the other HCD (anserine, homocarnosine), spanning the millimolar range. In some studies, the antioxidant effect of carnosine was compared with that of the constitutive amino acids, β-alanine and L-histidine, and the results depend on the models used, although it should be considered that β-alanine was usually found ineffective.

4. Antioxidant activity: in vivo models

The in vivo antioxidant activity of carnosine has been demonstrated in several studies which can be divided into two groups: the first, reporting the antioxidant efficacy of carnosine in physiological conditions, and the second, in animal models where oxidative stress was caused by xenobiotics acting as radical inducers, such as ethanol, thioacetamide, and anticancer agents.

In physiological conditions, carnosine was found to reduce, on one hand, the oxidative damage and on the other hand to improve the enzymatic and nonenzymatic antioxidant activity. More specifically, carnosine treatment for 1 mo at a daily dose of 250 mg/kg (ip) was found effective in aged (but not in young) rats to reduce some markers of lipid oxidation and to restore the depleted levels of blood glutathione (GSH) and basal activities of antioxidant enzymes such as SOD and glutathione peroxidase (GPX) (26). Accordingly, Kim et al. (215) reported that 6 wk of carnosine supplementation at a dose of 0.5% in the diet of rats was found to increase the activities of enzymatic antioxidants such as SOD and GPX. Furthermore, the supplementation induced a decrease in lipid peroxidation in serum, liver, and skin, and positively modified blood lipid profiles. In pigs, the addition of 100 mg/kg diet increased GPX, SOD, and catalase activities in plasma, liver, and muscle as well as SOD and GPX expression in muscle (242). In addition to affecting antioxidant enzymes, carnosine was also found to impact on low-molecular-weight antioxidants. Aydin et al. (26) reported that liver vitamin E was found to be significantly elevated in carnosine-treated aged rats. A sparing or regenerating effect of carnosine towards endogenous antioxidants was demonstrated in the liver of rats treated with carnosine or L-histidine. Both compounds increased the liver content of GSH and α-tocopherol (398). In contrast to the above-mentioned studies, Ibrahim et al. (192) reported that carnosine treatment for 15 days at levels of 200 or 1,000 mg/kg diet did not significantly affect the antioxidant and oxidative status in skeletal muscle, liver, and blood of rats.

Many studies have been reported on the antioxidant activity of carnosine in animal models where the oxidative stress condition in different organs was induced by xenobiotics. The protective effect of carnosine in liver was studied in a rat model of ethanol-induced chronic liver injury (237). Carnosine, as well as L-histidine, was found to dose-dependently inhibit the ethanol-induced oxidative damage and inflammatory mediators, and to increase catalase expression, GSH content, and GPX activity (237). The antioxidant activity in the liver and the reduction in plasma transaminases activities by carnosine in ethanol-treated rats was confirmed by Artun et al. (22) and Baykara et al. (55). The hepatic antioxidant activity of carnosine was also confirmed in a model of thioacetamide-induced liver cirrhosis (27).

Supplemented carnosine was also found to act as an antioxidant in the brain, as evidenced by decreased MDA and protein carbonyls induced by ethanol ingestion (284) and by protecting cerebral cytosolic SOD in two in vivo models of oxidative stress: hypobaric hypoxia in rats (150 mg/kg carnosine in drinking water before the hypoxic exposure) and in senescence-accelerated mice (100 mg/kg carnosine in drinking water for 12 mo) (347).

The antioxidant activity of carnosine has been shown in skeletal muscle. A 2-wk L-histidine supplementation period in rats can markedly increase the skeletal muscle carnosine content. When subjecting these rats to a Fe-nitrilotriacetate administra-
In addition to inhibiting oxidative damage and regulating the antioxidant enzymes, some studies have found a strong relationship between the antioxidant activity induced by carnosine treatment and the protection of the organ injury and dysfunction induced by the oxidative damage. In particular, carnosine pretreatment was found to prevent bleomycin-induced lung injury, fibrosis and, most notably, a complete abrogation of the mortality (92). The protective effect of carnosine was attributed to its ability to scavenge superoxide and peroxinitrite which are induced by bleomycin treatment. A beneficial effect of carnosine treatment was also reported in a model of cardiomyopathy induced by adriamycin which is a chemotherapeutic agent able to induce hydroxyl radical formation and lipid peroxidation (121). Finally, the antioxidant activity of carnosine was demonstrated in a model of kidney damage caused by cisplatin which is known to induce ROS in renal epithelial cells primarily by decreasing the activity of antioxidant enzymes and by depleting intracellular concentrations of GSH. Carnosine prevented the cisplatin-induced kidney oxidative damage, together with a protection of the organ histology and function (135, 274).

It can be concluded that there is much evidence indicating that, at least in rodents, supplemented carnosine acts as an antioxidant in both physiological conditions and in models of induced oxidative damage by xenobiotics. The antioxidant effect can be explained by considering both a direct scavenging effect towards ROS and by an increase and/or sparing and/or regenerating effect on enzymatic and nonenzymatic antioxidants. In some cases, such an antioxidant effect was found to be linked to a reduction in tissue damage and functional impairment. However, some questions remain unanswered, in particular regarding the mechanism through which carnosine potentiates the enzymatic antioxidants. Moreover, most of the studies showing the in vivo antioxidant activity of carnosine were carried out by supplementing carnosine at pharmacological doses (~100 mg/kg) for several weeks. It remains to be established whether endogenous carnosine (in nonsupplemented conditions) is contributing to the antioxidant system. Furthermore, it should be clarified in what body compartments this contribution is situated and whether the system is of quantitative relevance in the human organism.

D. Inhibiting Protein Carbonylation and Glycation

Several in vitro and in vivo studies have reported the ability of carnosine and related peptides to prevent the formation of advanced lipoxidation end-products (ALEs) and advanced glycoxidation end-products (AGEs). These compounds are both involved in the aging process as well as in the onset and propagation of several oxidative-based diseases such as diabetes, atherosclerosis, and Alzheimer’s disease.

1. In vitro models

Hipkiss first reported a series of papers showing the ability of carnosine to inhibit AGEs and ALEs formation induced by different precursors, including reducing sugars such as glucose (181), deoxyribose, ribose, fructose (180), and reactive carbonyls such as malondialdehyde (183, 184), glyoxal, methylglyoxal (179), acetaldehyde, and formaldehyde (182). The ability of carnosine to inhibit AGEs and ALEs formation has then been confirmed and extended in different in vitro models, as recently reviewed by Hipkiss (174). The ability of carnosine to prevent AGEs and ALEs formation is mediated by different mechanisms, and this is due to the fact that AGEs and ALEs formation is a quite complicated pathway, involving different reaction mechanisms and several catalyzing agents, including transition metal ions. Glycation represents the first step of AGEs formation, involving the reaction of the carbonyl group of reducing sugars with the amino group of lysine, followed by several oxidative steps that are catalyzed by metal ions. An alternative pathway consists of a direct reaction between reactive carbonyls generated by sugar auto-oxidation or AGEs decomposition and the nucleophilic sites of protein. A detailed description of the pathways of AGEs and ALEs formation is beyond the scope of the present review, but the reader is referred to some extensive reviews published on this aspect (11, 268, 285).

The different sites of the AGEs and ALEs cascade where carnosine has been proposed to act is shown in FIGURES 10 and 11, respectively. Carnosine was found to be effective already in the first step of AGEs formation and in particular in inhibiting protein glycation as well as by reversing glycated protein through a transglycation mechanism. In more detail, carnosine was found to protect glycation-induced loss of enzyme activity, and this effect paralleled the adduct formation between carnosine and sugar aldehyde such as glyceraldehyde 3-phosphate (330). The antiglycating property of carnosine has been found in several other in vitro methods (15, 295, 305, 312, 331, 397). Carnosine and anserine were also reported to act as transglycating agents, thus reversing the formation of the Schiff base, which is the first product of protein glycation (353). The mechanism is based on the nucleophilic attack of carnosine and/or anserine on the preformed aldosamine such as glucosyl-lysine with formation of glucosyl carnosine or anserine. The transglycation properties of carnosine have been confirmed in different in vitro models, the most striking being their ability to reverse pre-existing glycated proteins (332). Another potential mechanism of carnosine concerns its ability to detoxify reactive carbonyl species (RCS), precursor of AGEs, but up to now no direct evidence has been reported to confirm such mechanism. Carnosine can also inhibit AGEs formation through an antioxidant mechanism and more likely by a metal ion chelat-
ing mechanism as suggested by Nagai et al. (266), who showed that AGES inhibitors and breakers act primarily as chelators, inhibiting metal-catalyzed oxidation reactions that lead to AGES formation.

Carnosine is also a potent inhibitor of ALEs formation. There is currently enough evidence to indicate that this relates to the ability of carnosine to act by a direct RCS quenching mechanism (10). The ability of carnosine to react with α,β-unsaturated aldehydes, such as HNE, which represent the most abundant class of ALEs precursors, was firstly reported by Zhou et al. (407), who demonstrated the disappearance of the reactive aldehydes (measured through HPLC) when incubated in the presence of carnosine. By means of MS and NMR, the reaction mechanism was further elucidated by showing the quenching effect of carnosine and by identifying the structure of the reaction products (9, 12). Particularly, the N-acetylation of the β-alanine amine group was found to preclude the quenching activity. Moreover, the β-alanine and L-histidine were less efficient than carnosine itself, indicating that 1) the amino acids act synergistically in the dipeptide and 2) the reaction involves the amine group of β-alanine. As depicted in Figure 12, the multistep reaction mechanism between carnosine and HNE involves both the amino group of β-alanine and the imidazolic ring as reported by two independent groups (9, 238). In more detail, the reaction starts with the formation of a Schiff base that is formed by the reaction of the aldehyde with the amino group, followed by an intramolecular Michael addition, yielding the macrocyclic adduct which finally hydrolyzes to form the stable hemiacetal derivative (Figure 12, compound 5a). The reaction was then elucidated also for acrolein, which is another reactive α,β-unsaturated aldehyde (78). In addition to test tube experiments, the formation of the Michael adducts between carnosine and anserine with HNE were also demonstrated by LC-ESI-MS in biological matrices and in particular in oxidized skeletal muscle (283).

In addition to acting as an inhibitor of AGES and ALEs formation, carnosine was also hypothesized to act on preformed AGES/ALEs. Hipkiss found that carnosine is not only able to prevent protein carbonylation but also to react directly with protein carbonyl groups, producing protein-
carbonyl-carnosine adducts or “carnosinylated” proteins, thereby preventing cross-linking to other, unmodified protein (76, 176, 178). It was also suggested that since oxidized proteins can inhibit proteasome function, carnosine may suppress these inhibitory effects on proteasome activity and possibly facilitate their proteolytic elimination, through reacting with carbonylated proteins (“carnosinylation”).

2. In vivo models

The ability of carnosine to prevent AGEs and ALEs formation has also been demonstrated in some cellular models. By using a prokaryotic model (Escherichia coli), Pepper et al. (295) reported the ability of carnosine to suppress methylglyoxal- and glucose-mediated toxicity and the formation of AGEs such as carboxymethyl lysine (CML). In cultured neurons, Cheng et al. (82) demonstrated that MDA caused protein cross-linking and cytotoxicity, and that both these
effects were reversed by carnosine treatment. Carnosine was found to protect human peritoneal mesothelial cells against the toxic effect of glucose degradation products through the protection of cellular protein from modification and ROS-mediated oxidative damage (15). In addition to inhibiting AGEs formation, carnosine was also found able to protect cells against AGE-induced cell damage. In human skin fibroblasts, carnosine was more effective than N-acetylcysteine in avoiding cell damage induced by AGEs (302).

With respect to ALEs, carnosine has the capacity to quench HNE in vivo as demonstrated by identifying the HNE-carnosine Michael adduct and related metabolites in the urine of Zucker obese rats, which is an animal model of oxidative stress and carbonylation damage (282). Aldini et al. (14) recently reported that carnosine supplementation for 24 wk in Zucker rats significantly reduced the urinary AGEs and protein carbonylation in the kidney, and improved collagen solubility as an indicator of the extent of collagen cross-linking. However, more studies regarding the in vivo effect of carnosine on AGEs and ALEs formation should be carried out, and it remains to be understood whether the reaction of carnosine towards electrophilic aldehyde is catalyzed by an enzyme. Recently, some studies have shown that oral carnosine supplementation was not effective in reducing the levels of methylglyoxal-, CML-, and N-acetylglucosamine (304, 317).

E. Carnosine and Aberrant Proteins

Carnosine has been shown to stimulate neutral (nonlysosomal) protease activity in cell-free extracts from rat brain and in cultured cardiomyocytes (57, 71). Intracellular elimination of aberrant polypeptides mostly, though not exclusively, involves the proteasomes, and recent evidence suggests that nitric oxide can stimulate proteasomal activity (223, 363) and that carnosine can interfere with NO production (see below). Moreover, carnosine may accelerate protein turnover in two steps: a first rapid step which involves a localized thermal unfolding, and then a proteolytic event, which is slower (199). Possibly, the dipeptide can initiate the first step in protein removal, through assistance in damaged proteins unfolding. Carnosine enhances the thermal unfolding of glycated protein as evidenced by a decreased Gibbs free energy barrier and, additionally, decreases the enthalpy of denaturation, suggesting that carnosine may promote hydration during heat denaturation of glycated protein (399).

Carnosine was also reported to impact on aggregates and fibrils by disassembling/disaggregating them. This effect was observed by the group of Rizzarelli (24) showing that the dipeptide can prevent fibril formation in α-crystallin as well as in a more complex model of cultured lens organ. The lens transparency could be almost completely restored by disassembling already formed fibrils (24).
F. Carnosine and Nitric Oxide

Several studies have so far been reported on the ability of carnosine to impact on nitric oxide (NO) release, metabolism, and activity. There currently is an apparent contradiction in the literature, as some studies have shown a stimulating effect of carnosine towards NO production, while some others point to a direct or indirect inhibitory mechanism.

With regard to the stimulating effect, Alaghban-Zadeh (8) reported that carnosine, in the presence of NADPH, induced NO synthase (NOS) activity as measured by soluble guanylate cyclase. They identified that the effect was higher than that induced by arginine and β-alanine and comparable to that of L-histidine (8). More recently, Takahashi et al. (354) reported the ability of carnosine to stimulate NO production in the endothelium at concentrations higher than 5 mM, while neither the constituent amino acids of carnosine nor anserine had an effect on NO production. It was suggested that the mechanisms of carnosine action on eNOS activation may include carnosine-induced Ca^{2+} mobilization from intracellular stores. Carnosine was found to induce hyperactivity in chicks, and this effect was linked to NO generation via NOS in the brain, as demonstrated by using selective inhibitors as L-NAME (368).

The inhibitory effect of carnosine on NO-dependent signaling was first reported by Severina et al. (335), who showed that carnosine is a selective inhibitor of NO-dependent activation of guanylate cyclase. Later a direct scavenging effect of carnosine was demonstrated. In particular, in cell-free experiments based on competitive assays, the group of Rizzarelli (271) showed that carnosine significantly reduced NO concentration and that such a scavenging effect was not reproduced using the constituent amino acid of carnosine or their mixture, thus suggesting that the two amino acids act synergistically only when combined in the dipeptide. The scavenging activity of carnosine was then confirmed by ESI-MS, showing the formation of carnosine/NO and carnosine/NO₂ adducts at different ratios and the binding of L-histidine with NO (271). The scavenging activity of carnosine was then tested in a cell system, by examining the protection that carnosine provides against NO-induced cell death in primary rat astroglial cell cultures treated with lipopolysaccharide and interferon-γ. A correlation was found between cellular protection and NO free radical scavenging activity of carnosine. Also N-acetyl carnosine was demonstrated to directly react with NO (132). In conclusion, there is available evidence for both an inhibitory and stimulatory effect of carnosine on NO production and metabolism, and it remains to be further determined which factors (concentration dependence, tissue specificity, etc.) drive the effect in one way or the other.

IV. ENZYMES AND TRANSPORTERS INVOLVED IN CARNOSINE METABOLISM

This section discusses the enzymes involved in the direct formation and degradation of carnosine and its methylated analogs. Other enzymatic reactions, such as decarboxylation of carnosine (carnicine production) and acetylation of carnosine, are poorly understood and will not be treated. The proteins capable of transmembrane transport of carnosine will be described at the end of the section. The functional groups of the carnosine molecule required for recognition by carnosinases and carnosine transporters are displayed in FIGURE 13.

A. Carnosine Synthase

Soon after the discovery of carnosine (154) it became clear that β-alanine and L-histidine were the two constituent
aa amino acids (153) required for synthesis. Indeed, during ontogeny, the appearance of dipeptides is accompanied by an abrupt decrease in the content of l-histidine and β-alanine (61). Bauman and Ingvaldsen (54) showed that they were linked in a β-alanyl-l-histidine and not histidyl-β-alanine formation. In the 1950s, the biochemical properties of the carnosine synthesis reaction were further established by in vitro experiments on partially purified enzyme preparations of chicken muscle, and the enzyme was called carnosine synthase (CS). It became clear that in addition to the constituent amino acids, Mg²⁺, and ATP are required to synthesize carnosine (205, 391). CS appeared to have broad substrate specificity, and various other dipeptides can be formed by β-alanine or l-histidine replacement (205).

Over the next 50 years, several attempts were made to further purify the enzyme (189, 339, 345), but it was not until 2010 that CS was finally molecularly identified by Drozak et al. (114). This breakthrough was necessary to resolve and correct the reaction properties that were derived from previous experiments with less purified preparations. A 1,000-fold purification of chicken CS was shown to stoichiometrically form ADP + Pi, rather than AMP + pyrophosphate, as was previously suggested (205, 345). Characterization of CS as an ADP-producing ligase then led to the identification as a member of the ATP-grasp superfamily and more specifically as ATPGD1 (ATP-Grasp domain containing protein 1; Gencode: NP_001159694.1) (114). The enzyme is thought to be a homotetramer with a molecular mass of ~430 kDa for the native enzyme. Its gene is located on chromosome 11q13 in humans and contains 9 coding exons and expresses a 950-amino acid sequence.

Although early experiments (390) suggested that liver slices can synthesize carnosine, it later became clear that carnosine synthase (ATPGD1) is mainly present in skeletal and heart muscle and certain brain regions (114). The olfactory neurons display very high CS expression, which is in agreement with its very high carnosine content (189, 248). In other brain structures, however, homocarnosine rather than carnosine is the main dipeptide. CS is now believed to be responsible for the homocarnosine synthesis in the brain (269, 339). Oligodendrocytes, but not astrocytes, have been shown to possess carnosine synthase activity (51, 186).

The enzyme is localized intracellularly, and studies on brain subcellular fractions indicate that it is a cytosolic enzyme (161, 270). The targetP calculation of the protein sequence, which predicts the subcellular location of eukaryotic proteins, confirms the expected cytosolic localization (114). Little is known about the regulatory mechanism of CS activity and expression. The highly conserved cysteine residues in the carnosine synthase protein sequence suggest that the enzyme is potentially redox regulated (114).

Recently, Tsubone et al. (375) reported a potential novel imidazole dipeptide synthase, which they purified from eel muscle. They propose that this enzyme is different from the above-mentioned CS (EC 6.3.2.11), as the synthesis would not require ATP.

### B. Carnosine N-methyltransferase

There are two possible pathways for the synthesis of the methylated carnosine analogs, anserine (β-alanyl-Npi-methyl-histidine) and ophidine/balenine (β-alanyl-Ntau-methyl-histidine). The first enzymatic pathway is through carnosine N-methyltransferase (CMT), which catalyzes the transfer of a methyl group of S-adenosylmethionine (SAM) on carnosine to form anserine (or ophidine). A second pathway is the enzymatic condensation of β-alanine with Npi-methylhistine (or Ntau-methylhistidine) through CS. Several studies suggest that the former pathway, i.e., through CMT, is of more physiological importance.

The first evidence for the existence of a carnosine methylation process was provided by Winnick and Winnick (391), who demonstrated in vitro in a chick pectoral muscle preparation that a methyl group from SAM can be transferred to carnosine. Subsequently, McManus (254) partially purified CMT (EC 2.1.1.22) from this chick pectoral muscle preparation. The Kₘ for carnosine was found to be 4 mM, which is a concentration comparable to the endogenous muscle levels. The enzyme is confined to the soluble fraction of muscle homogenate, and it does not seem to have specific ion requirements. Interestingly, l-histidine was only found to be a substrate of this enzyme preparation, when bound into carnosine, and not as a free amino acid. Raghavan et al. (310) showed that CMT can also methylate l-histidine residues in proteins, in conjunction with another unidentified protein, histidine-N-methyltransferase (EC 2.1.1.85). l-Histidine methylation in proteins is a rare but evolutionarily conserved posttranslational modification that specifically occurs in muscle proteins actin and myosin.

The second possible pathway for anserine (ophidine) synthesis is through direct condensation of β-alanine with
methyl-L-histidine. The ability of CS to use methyl-L-histidine as a substrate has been demonstrated by in vitro enzymatic assays with chicken muscle preparations by two independent groups in 1959 (205, 391). This was recently confirmed using recombinant CS (114). Yet, methyl-L-histidine was consistently found to be a poorer substrate than nonmethylated L-histidine, making anserine synthesis slower than carnosine synthesis. Furthermore, a study by Bauer and Schulz (53) in primary culture myocytes indicated that anserine is not directly formed by condensation of radiolabeled β-alanine with Npi-methyl-L-histidine, but rather as a consequence of carnosine methylation, secondary to carnosine synthesis. Thus CMT and not CS is the major enzyme involved in anserine synthesis.

Drozak et al. (113) recently molecularly identified the avian CMT as the histamine N-methyltransferase (HNMT)-like protein. Although the enzyme has high specificity for carnosine as a methyl acceptor, it probably evolved as a paralog of a histamine-methyltransferase, which acquired new enzymatic activity (113). Surprisingly, the mammalian genomes do not contain orthologs of the CMT gene (HNMT-like), discovered in chicken, meaning that the mammalian version evolved in a different manner and still needs to be discovered.

There is little or no insight into whether there are further processes regulating anserine rather than balenine/ophidine synthesis, but it would make sense that there is a different enzyme for each compound. Likewise, it is unclear whether the acetylation of carnosine, anserine, and homocarnosine requires a specific enzyme to make the specific products (acetylcarnosine, acetylanserine, etc.) present in cardiac tissue (279, 280).

C. Carnosinas

Carnosine and related compounds are not degraded by regular (di)peptidases, but their metabolism is characterized by its own hydrolytic enzymes, named carnosinas. Carnosinase was first described and partially purified by Hanson and Smith in 1949 from swine kidney (158). Two forms have since been molecularly identified as CN1, or serum carnosinase, and CN2, or tissue carnosinase or cytosolic nonspecific dipeptidase (229, 360). They are members of a family of M20/M28 metalloproteases. CN1 and CN2 show 53% sequence identity in humans, and both enzymes exist as homodimers. A recent crystallographic study revealed that each subunit of the carnosinas consist of two domains, an A domain with catalytic and metal binding activity and a B domain for dimerization (376). The genes of these carnosinas are CNDP1 and CNDP2, and they are localized side by side on human chromosome 18 in a head-to-tail position.

1. Human serum carnosinase

Human serum carnosinase or CN1 (EC 3.4.13.20) was first described in relation to carnosinemia, a disorder associated with nutrition-independent elevated circulating carnosine levels and a mental defect in children (297) (see sect. VII). Under nonpathological conditions, serum carnosinase activity is high in the adult human, leading to almost undetectable levels of circulating carnosine in the postabsorptive state (143). The activity of serum carnosinase (CN1) varies greatly between individuals. CN activity is very low shortly after birth and gradually increases to adult levels during adolescence, and attains higher levels in females than males (126, 301).

CN1 is highly present in the serum of humans, but absent from nonprimate mammals, except for the Syrian golden hamster (196). For its presence in human serum, the enzyme may be produced by the brain, but is presumably mainly produced and secreted from the liver into the circulation, which is supported by the finding that patients with liver cirrhosis have low CN1 activity (300). By analogy with other secreted proteins, CN1 is synthesized as a precursor containing an NH2-terminal signal peptide sequence. There is a (CTG)n polymorphism in this signal peptide which appears to determine the rate of CN1 secretion. Riedl et al. (315) recently showed that a high number of leucine repeats enhances the secretion efficiency of carnosinase to the serum (see sect. VII). In addition, CN1 is N-glycosylated at three different sites, and the glycosylation appears to be essential for secretion of the enzyme (316). Anserine, ophidine, and homocarnosine are found to be more resistant to carnosinase (293) and to be potent inhibitors of carnosinase activity towards carnosine as a substrate (300, 301).

2. Cytosolic nonspecific dipeptidase

According to Teufel et al. (360), carnosine is only a substrate of cytosolic nonspecific dipeptidase or CN2 (EC 3.4.13.18) under nonphysiological conditions (pH optimum of 9.5). Therefore, the authors propose that CN2 is renamed as cytosolic nonspecific dipeptidase. However, a more recent report suggests that, even though the catalytic rate of CN2 is markedly lower than that of CN1, carnosine can still be hydrolyzed by CN2 under physiological conditions (287). It remains to be determined whether CN2 is involved in the degradation of carnosine in tissues with abundant carnosine content and in the intestinal wall.

3. Substrate specificity and functional differences of CN1 and CN2

TABLE 1 summarizes the main functional differences between CN1 and CN2. CN2 has a broader substrate specificity than CN1, but it does not hydrolyze homocarnosine. In contrast to CN1, CN2 activity is inhibitable by bestatin,
which is a substrate mimic of carnosine. Another marked difference with other family members of M20/M28 metallopeptidases, which are all activated by Zn$^{2+}$, is that CN2 requires Mn$^{2+}$ for its catalytic activity.

4. Other dipeptidases with carnosinase activity

Anserinase or Xaa-methyl-His dipeptidase (EC 3.4.13.5) is a dipeptidase found in fish, with a broad substrate specificity, including carnosine, anserine, and homocarnosine. It was named anserinase because it was first described in codling muscle (203), which uniquely contains anserine as HCD. Yamada et al. (394) recently identified anserinase molecularly and proposed a phylogenetic tree with three separate families of carnosinase-like enzymes, being serum carnosinase-like (CN1), cytosolic nonspecific dipeptidase-like (CN2), and anserinase-like. Other carnosinases have been described within the EC 3.4.13 family of dipeptidases, but their enzyme classification (such as EC 3.4.13.3) is now deleted because its activity is covered by CN1 and CN2.

A handful of carnosinas/β-peptidyl aminopeptidases have now been described in bacteria, and this new peptidase family is rapidly expanding (145). One of the first described bacterial enzymes that can hydrolyze carnosine is the dinuclear zinc aminopeptidase (PepV), characterized in Lactobacillus delbrueckii (385). Similarly, l-aminopeptidase D-Ala-esterase/amidase (DmpA) from Ochrobactrum anthropi, β-alanyl-Xaa dipeptidase (Ps BapA) from Pseudomonas sp. and BapF from Pseudomonas aeruginosa show high specificities towards carnosine (138, 145). Aminoacyl-histidine dipeptidase (also named Xaa-His dipeptidase, carnosinase, or PepD) has recently been cloned from the bacterium Porphyromonas gingivalis (18). The recombinant protein exhibits carnosinase activity, which is dependent on transition metals, such as Cu$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$. P. gingivalis is associated with periodontal disease and rheumatoid arthritis, for which carnosine has been suggested to have therapeutic potential (110). It remains to be determined whether the carnosinase activity of these bacteria is of pathogenic relevance and how this interacts with the carnosine content in human tissues.

D. Carnosine Transporters

Carnosine can be transported across the cellular membrane through a number of transporters from the proton-coupled oligopeptide transporter family (POT-family or SLC15). The mammalian members of this family are PEPT1 and PEPT2 (oligopeptide transporter 1 and 2) and PHT1 and PHT2 (peptide/histidine transporter 1 and 2). All of these POTs have a broad specificity and are able to transport carnosine and its methylated analogs (395). In addition, they can transport a high number of other peptides. PEPT1 and PEPT2 are believed to have the capability to transport 400 different dipeptides and 8,000 different tripeptides, but not longer peptides or single amino acids. Whether the specificity of PHT1 and PHT2 is equally broad is less clear, but the main difference with the PEPTs is that the PHTs can transport l-histidine, in addition to di/tripeptides. The physiology and pharmacology of the SLC15 transporter family has been reviewed by Daniel et al. (93, 94). The following section will mainly review the literature on the functional transport of carnosine by POTs in human tissues.

1. PEPT1 and PEPT2

PEPT1 is characterized as a high-capacity, low-affinity transporter and plays a major nutritive role in the intestinal absorption of peptides (FIGURE 14). Carnosine can be absorbed in the small intestine, and at least part of it enters the blood intact (not hydrolyzed) upon oral ingestion (49, 143). Carnosine uptake across the brush-border membrane is accomplished by PEPT1. When carnosine enters the enterocytes, it is either hydrolyzed, presumably by carnosinase (CN2), or further transported across the basolateral membrane. PEPT1 is a peptide/proton cotransporter, and its activity is therefore increased with a lower luminal/apical pH.

<table>
<thead>
<tr>
<th>Table 1. Comparative table of characteristics of dipeptidases with carnosinase activity</th>
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<tr>
<td><strong>Other names</strong></td>
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<tr>
<td><strong>Expression in human tissues</strong></td>
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<tr>
<td><strong>Subcellular localization</strong></td>
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<tr>
<td><strong>Metal ion cofactor</strong></td>
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<tr>
<td><strong>Inhibitable by bestatin</strong></td>
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<tr>
<td><strong>pH optimum for carnosine hydrolysis</strong></td>
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<td><strong>Substrate specificity</strong></td>
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Based on data from Peters et al. (301), Pegova et al. (293), and Lenney et al. (230).
PEPT2 is characterized as a low-capacity, high-affinity transporter and contributes to reabsorption of filtered peptides in the renal tubule, where it is mainly localized in the apical membrane of epithelial cells. Insight into the role of PEPT2 in carnosine metabolism has been advanced by the development of PEPT2 knockout mice (320). With respect to kidney function, PEPT2 ablation markedly impedes the tubular reabsorption of carnosine, as the renal carnosine clearance is close to the glomerular filtration rate in the null mice, whereas it is 20-fold lower in the wild-type mice (206). Even though the carnosine uptake is high in the apical side of the epithelial cells, its further transport to the blood through the basolateral membrane is very limited (198). This means that carnosine, once arrived in the epithelial cytosol partly accumulates but is mainly hydrolyzed by carnosinase, after which the constituent amino acids are transported to the blood over the basolateral membrane by amino acid transporters. Thus, even though the dipeptide transporter (PEPT2) plays a determining role in renal carnosine reabsorption, the transepithelial flux of carnosine is limited and the integrity of the dipeptide is probably lost during the renal passage.

In addition to its designation as, respectively, the intestinal and renal isoform, PEPT1 and PEPT2 are expressed in several other (human) tissues. In the choroid plexus, PEPT2 is responsible for over 90% of the carnosine uptake from the CSF over the apical membrane into the epithelial cells (361, 362). This suggests that PEPT2 plays a critical role in the homeostasis of carnosine (and other peptides) in the CSF. Also other glia, such as astrocytes, seem to primarily depend on PEPT2 for their uptake of carnosine (393). Equally, other cell types such as cardiomyocytes (233) and epithelial tissues (human nasal epithelium) have been shown to express PEPT2, along with the other POTs, and to possess the ability to transport carnosine (6). The latter finding may have importance as a potential route to administer carnosine or carnosine-related drugs.

2. PHT1 and PHT2

Whereas good biochemical and physiological information is available for the PEPTs, relatively little is known about the more recently discovered PHTs. The first PHT (PHT1) was cloned from rat brain by Yamashita et al. (395). When expressed in *Xenopus laevis* oocytes, carnosine transport...
Physiol Rev • VOL 93 • OCTOBER 2013 • www.prv.org

activity of PHT1 was confirmed. Bhardwaj et al. (58) similarly cloned human PHT1 (hPHT1) and transfected it in COS-7 cells, in which it elicited pH-dependent carnosine transport. The second peptide/L-histidine transporter (PHT2) was identified by Botka et al. (72) and Sakata et al. (321). Recombinant rPHT2 protein reconstituted into liposomes displays transport activity towards carnosine (321).

Northern analysis indicated that hPHT1 is primarily expressed in skeletal muscle and spleen, whereas hPHT2 is found in spleen, placenta, lung, leukocytes, and heart (72). As the majority of carnosine in the human body is found in the skeletal muscle compartment, it is expected that carnosine transporters are functionally expressed in skeletal muscle cells. Everaert et al. (125) recently demonstrated that mRNA transcripts of the PHTs but not the PEPTs were found in mouse and human skeletal muscle samples. It is currently unclear what their physiological function is in muscle and whether inward or outward transport is the main direction.

V. PHYSIOLOGICAL ROLE OF CARNOsINE

In this section, we focus on the putative roles of carnosine and related compounds in different organs and organ systems. We limit our discussion to tissues in which carnosine content is high or at least evidence for its involvement in normal physiological function is available, and this seems to mainly be the excitatory tissues, skeletal and cardiac muscle, and neuronal tissue. It is evident that carnosine does not have the same function in different tissues, and even may fulfill different functions within one tissue. There is little clarity about the role of endogenous carnosine in tissues, other than the nervous system and skeletal and cardiac muscle. This probably relates to the fact that there is still controversy as to whether carnosine is endogenously present in organs such as the liver and kidney (see sect. II).

A. Carnosine in Skeletal Muscle Function

From the very beginning, the research on the physiological role of carnosine was directed towards skeletal muscle, as the molecule was initially discovered in meat extract. Yet, over a century of research has not yet led to the establishment of the definitive role of this compound in the tissue in which it is so abundant. Several hypotheses were proposed, and some were subsequently refuted, such as the idea of carnosine functioning as a high-energy phosphate system, similar to the creatine/phosphocreatine system (148).

Severin, one of Gulewitch’ pupils (334), suggested that carnosine plays a role in the contractile function of skeletal muscle. In their experiments they demonstrated in nerve-muscle preparations of frogs that the addition of 10 mM carnosine to the surrounding medium can offset the fatigue that occurs during rhythmic nerve-stimulated muscle con-
tractions. This anti-fatigue effect of carnosine was subsequently adopted as the “Severin’s phenomenon,” which also holds true for anserine (64). Below we summarize the currently proposed physiological roles of carnosine in skeletal muscle, depicted on FIGURE 15.

1. Intramyocellular homeostasis during contractions

The role of carnosine as a pH buffer in skeletal muscle was first proposed in 1938 by two independent groups (47, 107). Bate Smith (47) proposed that carnosine would account for up to 25% of the buffering capacity in rigor muscle and as much as 40% in vivo. During high-intensity muscle contractions, the anaerobic glycolysis leads to the production of lactic acid which immediately dissociates into protons (H\(^+\)) and lactate ions at physiological pH values. The resulting acidosis can reach pH values of 6.5 and lower (191) and has often been associated with muscle contractile fatigue. Even though the role of acidosis in muscular fatigue remains a matter of debate (16, 292), it can be expected that the reason for the abundance of carnosine in muscle is at least partly related to the proton-sequestering property of the carnosine molecule (see sect. III).

In addition to HCD, several compounds, such as proteins, bicarbonate, and inorganic phosphate, contribute to the cellular buffering system, and their relative contribution markedly depends on the muscle types and animal species (281, 381)(see sect. II). For instance, the HCD may account for more than half of the total muscle buffering capacity (β\(_{\text{tot}}\)) in marine animals, such as whale, who are involved in prolonged hypoxic dives (3). HCD is typically high in white (fast-twitch) muscle of animals in which anaerobic exercise types such as prolonged breath-hold diving and sprint swimming, running, flying, and hopping are required to catch prey or escape predators (3).

In humans, muscle contains a rather small amount of HCD (see sect. II), which would mean that they contribute relatively little to β\(_{\text{tot}}\). The relative buffering capacity \(\beta_{\text{carnosine}}/\beta_{\text{tot}}\% \) in the human vastus lateralis muscle was determined to be 4.5 and 9.4% in fibers I and II, respectively (243). When muscle carnosine content is increased by nutritional intervention, the degree of acidosis in the blood during high-intensity exercise will be attenuated in humans (38). Possibly more important than its contribution to β\(_{\text{tot}}\) carnosine is a mobile buffer, freely dissolved in the myoplasm, as opposed to proteins, which are fixed buffers (204, 340). This means that its contribution to pH homeostasis and prevention of local pH gradients is probably greater than would be expected from its calculated proportion of β\(_{\text{tot}}\).

It seems likely that the physiological role of carnosine in contracting muscle relates to homeostasis, but this may include more than only protection against acidosis. One such alternative perturbation may be the protection against exercise-induced production of ROS in muscle. The evidence for car-
nosine’s antioxidative function in muscle in vivo is very limited at present. A study on 90-min downhill running in rats showed that a supplementation-induced increase in muscle HCD levels could diminish the production of thiobarbituric acid reactive substances (TBARS, lipid peroxidation products) (97). This study is in line with earlier Russian studies showing a protecting role of carnosine on lipid peroxidation in working muscles (67). Another putative role of carnosine in skeletal muscle tissue is as a physiological chelator of Cu\(^{2+}\). Schröder et al. (329) have demonstrated the presence of Cu\(^{2+}\)-carnosine chelates in human calf muscle by proton MR spectroscopy in vivo. However, there are currently no data available to confirm or refute the hypothesis put forward by Brown in 1981 on the regulation of anaerobic and aerobic metabolism through Cu\(^{2+}\)-carnosine complexation (74).

2. Regulation of excitation-contraction coupling

The excitation-contraction coupling in skeletal muscle cells is a complexly regulated chain of events in which calcium is released from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR), the released calcium will bind to troponin C to allow cross-bridge formation and force production and ultimately the reuptake of calcium in the SR will terminate contraction (150). Carnosine has been suggested to be involved in the regulation of several of these steps (FIGURE 15).

Batrukova and Rubtsov (48) demonstrated in SR vesicles from rabbit skeletal muscle that carnosine can induce Ca\(^{2+}\) release, whereas its constituent amino acids L-histidine and \(\beta\)-alanine, added alone or in combination, were not effective. Similar evidence for carnosine’s potentiating effect on SR calcium release was presented in a study on chemically skinned fibers of human vastus lateralis muscle (404). However, these conclusions were contested by Dutka and Lamb (122), arguing that all previous studies were performed with abnormally low Mg\(^{2+}\) levels. They instead demonstrated that carnosine does not play a role in stimulating the RyRs in the presence of physiological levels of Mg\(^{2+}\) (122).
More recently, however, Dutka et al. (123) demonstrated in mechanically skinned human muscle fibers that carnosine in a physiological concentration can sensitize the RyRs to Ca\(^{2+}\)-induced Ca\(^{2+}\) release in type I, but not type II, fibers.

A step that is probably even more important for carnosine’s effect on excitation-contraction coupling is the calcium sensitivity of the contractile apparatus. Dutka and Lamb (122) demonstrated that physiological carnosine concentrations (4–16 mM) improve Ca\(^{2+}\) sensitivity of rat extensor digitorum longus (EDL) fibers in a concentration-dependent manner, hereby confirming previous work from Lamont and Miller (227) on frog sartorius muscles. More recently, these observations were also demonstrated in skinned human muscle fibers (123). In the fast-twitch fibers, the improved calcium sensitivity probably entirely explains the force-potentiating effect of carnosine, as this fiber type did not show improvement in calcium release (123). Not only in skinned fiber preparations, but also in whole incubated skeletal muscles of mice, force potentiation in response to nutritionally elevated muscle carnosine levels was observed, based on a leftward shift of the force-frequency relationship (127).

3. Muscle as a depot and donor of carnosine

It is tempting to speculate on the possible role of skeletal muscle as a production, storage, and release organ for carnosine. Indeed, muscle contains the vast majority of the carnosine present in the organism, and it is one of the few tissues where the expression of the enzyme responsible for carnosine synthesis is very high. Early in the scientific exploration of carnosine’s function, Russian scientists came to the conclusion that tissues that are capable of synthesizing carnosine should not be able to provide for its degradation (64). Indeed, tissues and cell types either have a high capacity to synthesize carnosine (through CS) or to degrade carnosine (through CN), but never combine both capacities. This suggests that tissues, such as skeletal muscle, that synthesize carnosine can also release it to other places where it can be degraded/utilized, e.g., for delivery of L-histidine or β-alanine. Thus muscle carnosine could play an autocrine, paracrine, or even an endocrine role.

There is some evidence to believe that especially during exercise, thus when skeletal muscles contract, they release carnosine into the interstitium and the circulation. This has been shown in rats by Nagai et al. (262), who observed a doubled carnosine concentration in plasma of rats who had access to a running wheel compared with sedentary rats. Similar, though smaller, changes were observed in running horses (119). Also in human skeletal muscle, carnosine content markedly increases in the interstitial fluid during knee extension exercise at 20W, determined by microdialysis (275). However, it is not known whether this carnosine release is substantial enough to elevate human plasma carnosine levels during and following exercise. The fact that not only carnosine, but also the L-histidine, concentration increases in skeletal muscle interstitium in humans (evaluated by microdialysis; Ref. 156) would indicate that a portion of the released carnosine is promptly hydrolyzed by serum CN1. There is controversy as to whether carnosine release by muscle cells is a result of either sarcolemmal rupture and microtrauma, or of a functional transporter-mediated process. In belief of the former, some researchers have interpreted the release of carnosine by exercising muscles as a measure of exertional rhabdomyolysis and muscle damage (119, 156, 275). Others, however, believe that myocytes actively release/secrete carnosine through dipeptide transporter, which are expressed in muscle (see sect. IV) as part of a physiological regulatory process. The physiological function of such contraction-induced release mechanism is still elusive but could be related to the carnosine histidine-histamine pathway (151) (Figure 15).

The above-described functions are most likely the primary roles of carnosine in skeletal muscle. A number of other potential roles, e.g., activation of myosin ATPase, effects on glycolysis, mitochondrial respiration, and the neuromuscular junction, have been sporadically suggested, but remain poorly understood. A complete description of these studies is provided in the monograph of Boldyrev (61).

B. Carnosine in Brain Function

Homocarnosine is a more prevalent dipeptide than carnosine in the mammalian brain. This probably relates to the fact that next to L-histidine the other constituent amino acid of homocarnosine is GABA, which is the most important and widespread inhibitory neurotransmitter in the mammalian brain. However, also β-alanine, GABA’s equivalent in carnosine, is found in mammalian brain in concentrations ranging from 40 to 100 μM in various rat brain structures (103), and has been proposed to possess the properties of a neurotransmitter (364). The highest carnosine concentrations in the brain, found in the olfactory system, are comparable to those usually found in skeletal muscle.

In humans, brain homocarnosine concentration is rather high (0.5–1 mM) and detectable by proton magnetic resonance spectroscopy (MRS) in vivo (303). Carnosine is probably not detectable in the human brain by MRS, and its presence is presumably mainly limited to the olfactory system.

1. Distribution

The first evidence for the presence of carnosine in the brain was provided by Margolis, who demonstrated that the olfactory tract of mice contains significantly higher carnosine concentrations (1–2 mM) than other brain structures, where its concentration is usually ~0.1 mM or lower (246).
The olfactory system of many animal species, including humans, is highly enriched with carnosine as well as with its principal enzymes. Carnosine synthase activity is 50- to 100-fold higher in the olfactory epithelium than in brain structures and 10-fold higher than in skeletal muscle (160, 216).

Carnosine is located in the olfactory receptor neurons, more specifically in the perikarya and cell processes, including the axons and synaptic terminals in the olfactory bulb (59). When radiolabeled β-alanine and l-histidine are administered to the nasal mucosa, carnosine is rapidly formed and directed by axoplasmic transport to the olfactory bulb (247). Carnosine synthase activity in primary olfactory neurons decreases upon denervation and recovers upon regeneration (159, 160). These data indicate that the sensory neurons are primarily responsible for the presence of carnosine in olfactory epithelium and the olfactory bulb (98). Carnosinase, on the other hand, is mainly present in other cells, such as pillar cells and Bowman gland cells, of the nasal mucosa, which confirms the dogma that the enzymes of carnosine synthesis and degradation are located in different cell types and compartments (81, 159).

The olfactory neurons seem to be the only neurons that express carnosine. Other cells of the neuronal lineage, the neuroblasts of the subependymal layer (SEL) of the forebrain, have been reported to display carnosine-like immunoreactivity. However, the immunohistochemical results when using anti-carnosine serum may not allow to distinguish between the presence of either carnosine or homocarnosine (70, 98).

Carnosine-like immunoreactivity is widespread in glial cells throughout the brain. Carnosine synthesis activity was first demonstrated in cultured rat C-6 glioma cell lines (52). In mouse glial primary cultures, Bauer et al. (51) demonstrated that β-alanine is effectively incorporated into carnosine, whereas GABA was rapidly degraded. Similar carnosine synthesis activity could not be observed in neuronal cell cultures (50), but seems to be specifically confined to oligodendrocytes (186). Not only do glial cells display carnosine synthesis activity, they also possess the ability to transport carnosine, as observed by the release of significant amounts of synthesized carnosine from cultured glia into the medium. This carnosine transport activity seems to be primarily observed in astrocytes (186) and is probably operated by PEPT2 (112). Bauer (50) recently proposed that PEPT2, expressed in choroid plexus epithelial cells, is also involved in the removal of carnosine and other peptides from the cerebrospinal fluid (CSF).

2. Putative functions of carnosine in the brain

It is fair to say that we currently have a poor understanding of the role of carnosine in the brain. However, a number of potential roles have been proposed, and the evidence for these is summarized below.

On the basis of its high concentrations found in the olfactory neurons, carnosine was hypothesized to be involved in sensory neurotransmission, either as a neurotransmitter or a neuromodulator. As for most other sensory pathways, glutamate is the main excitatory neurotransmitter involved in the synapse between olfactory neurons and target cells (mitral and periglomerular cells) in the olfactory bulb. Interestingly, immunohistochemical studies have identified colocalization of carnosine and glutamate in the synaptic terminals of mouse olfactory neurons (324), as well as bipolar cells of the frog retina (288). This supports the hypothesis for a putative role of the dipeptide in neuromodulation of glutamatergic sensory neurons. However, there are a number of findings arguing against a direct role of carnosine in glutamatergic neurotransmission, which are summarized by Bonfanti et al. (70) and De Marchis et al. (98). Possibly, interaction between carnosine and glutamate involves glia. Bakardjiev (40) showed that oligodendrocytes exhibit glutamate-receptor mediated release of carnosine. More recently, Baslow (46) proposed that carnosine, homocarnosine, and other brain dipeptides such as N-acetylaspartylglutamate (NAAG) serve as neurotransmitters in neuron-to-glia communication.

In addition to its involvement in modulation of neurotransmission, the presence of carnosine in certain brain structures has been hypothesized to be homeostatic and protective, mainly through antioxidant, metal chelating, and antiglycative properties. It has been argued that the presence of other endogenous antioxidant systems (glutathione, vitamins C and E) availability is very low in the nervous system, which is compensated for by (homo-)carnosine (70). It seems clear that this aspect is of therapeutic value (see further in sect. VI), but it remains to be established whether this is a true physiological role in normal function as well. Carnosine has also been suggested to be indirectly involved in neurotransmission through its capacity to chelate transition metals such as copper and zinc, which in itself have been identified as modulators of neurotransmission (60, 373).

C. Carnosine in Cardiovascular Function

One of the first described physiological effects of carnosine was the transient decrease in systemic blood pressure upon intravenous injection of carnosine in dogs (313). The hypotensive and antihypertensive effect has since been frequently confirmed in various other mammalian species (115, 272). The lowering effect of carnosine on blood pressure is probably realized by a systemic arterial vasodilation. The direct vasorelaxing effect of carnosine has been demonstrated on isolated rat aortic rings (318). Interestingly, carnosine can also potently provoke sustained contractures in rabbit sa-
phenous vein rings (278). Given the potency of carnosine in the regulation of the vascular tone, it is thought that not only exogenously administered carnosine, but also endogeneously available carnosine takes part in normal hemodynamic regulation. The formation in vivo of a zinc/carnosine complex and the involvement of histamine H₁ receptors appear to be involved in vascular smooth muscle contraction/relaxation (255, 277). However, alternative vasoactive mechanisms of carnosine may be equally involved, such as the carnosine-histidine-histamine pathway (151), a NO/cGMP mechanism (318), and the modulation of the autonomic nervous system (265).

Carnosine can markedly potentiate the cardiac contractility, as demonstrated in experiments with isolated rat hearts (319). In parallel with the mechanisms underlying improvement of contractility in skeletal muscle, it is believed that also for the cardiac muscle the main mechanisms are improved calcium handling (excitation-contraction coupling) and increased pH buffering capacity. In chemically skinned cardiac myocytes, carnosine was able to both release calcium from the SR as well as improve the tension of the contractile proteins in response to calcium (403). Besides modulating intracellular calcium, it is likely that carnosine and other HCD play a role in the pH regulation and contribute to the mobile buffering system in the cardiac cell (380). In this respect, an interesting novel hypothesis was recently proposed by Swietah et al. (352), in which they describe carnosine as a diffusible Ca²⁺/H⁺ exchange “pump,” creating functional Ca²⁺ gradients in response to local pH changes in cardiomyocytes. Despite the above-mentioned studies, it is surprising to note that still so little scientific knowledge has been collected on the role of HCD in the cardiovascular system.

VI. CARNOSINE AND DISEASE

A. Extrapolation of Animal Models of Disease to Humans

With the exception of a limited number of small-scale clinical trials that have not been independently confirmed, such as on patients with Parkinson disease (62), schizophrenia (83), autistic spectrum disorder (84), and ocular diseases (30), the vast majority of research on the therapeutic potential of carnosine is conducted in animal models of human diseases, mainly in rodents. One should be aware, however, that there currently still is a potential problem with the transposability of these findings to humans. The limitation relates to the low levels of serum carnosinase activity in rodents compared with the high levels of carnosinase activity in humans. It is possible that a least part of the beneficial and therapeutic effects of carnosine administration is related to elevated plasma carnosine levels in animals, which are more difficult to obtain in humans (128, 400).

The metabolic instability of carnosine in human serum due to the presence of carnosinase has prompted a great interest in the design and chemical synthesis of novel carnosine derivatives, as well as in research towards the therapeutic potential of the natural carnosine derivatives, such as anserine (224). These compounds are more resistant to carnosinase, while maintaining or even improving some beneficial effects of the parent compound, such as the antioxidant and metal ion chelating ability or the reactivity with RCS. Various reviews have recently been published regarding the carnosine derivatives stable to carnosinases (56, 152, 383).

B. Diabetes

A growing body of evidence indicates a protective role of carnosine in diabetes due to its ability to affect glycemic control and to prevent/ameliorate diabetic complications such as nephropathy and ocular damage. An overall protective effect of carnosine in diabetic animal models was first published by Lee et al. (228) using Balb/cA mice. An oral supplementation with carnosine for 4 wk dose-dependently reduced plasma glucose and fibronectin levels, increased the insulin levels, and reduced the oxidative damage. A similar protective effect was induced by L-histidine, which was found to increase the carnosine content in plasma and organs to a similar extent to that reached by the carnosine treatment. The beneficial effects of carnosine on diabetic deterioration were confirmed some years later by Soliman et al. (341) in a streptozotocin diabetic-induced model. Carnosine treatment (100 and 200 mg/kg) dose-dependently reduced hyperglycemia, normalized dyslipidemia, and reduced liver damage. In addition to these beneficial effects, it should be noted that the papers by Lee and Solima both demonstrate a hypoglycemic effect of carnosine in diabetic rats. The ability of carnosine to control blood glucose levels was thoroughly investigated by Nagai et al. (262) who proposed that such effect occurs through the ability of carnosine, which is a precursor of histamine, to regulate the autonomic nervous system via the H₁ receptor. A confirmation of such a receptor-mediated mechanism comes from the similar hypoglycemic effects evoked by L-histidine and histamine. In more detail, carnosine lowers neuronal activities of sympathetic nerves and facilitates those of parasympathetic nerves causing both an increase in the insulin secretion and suppression in the glucagon secretion from the pancreas, resulting in a hypoglycemic effect. A similar hypoglycemic effect was more recently reported for anserine, which was also found to reduce blood glucose levels in humans during oral glucose tolerance testing (224). A direct correlation of carnosine serum levels with insulin levels was also detected by Sauerhoefer et al. (326) in db/db and hCN1 transgenic db/db mice, whereas peripheral insulin sensitivity was unaltered. The mechanism by which serum carnosine levels affect insulin secretion was addressed to the ability of carnosine to preserve or increase β-cells within the pancreas.
Carnosine was found to be effective in inhibiting the development of diabetic complications including ocular diseases and nephropathy (304, 317). Carnosine treatment was found to reduce diabetes-induced nephropathy and in particular to restrain glomerular apoptosis, to prevent podocytes loss and to reduce the expression of Bcl-2-associated X protein (Bax) and cytochrome c (317), well-known pro-apoptotic proteins. Carnosine was also found to ameliorate diabetic neuropathies, especially abnormal sensory perception (207).

Taken together, the protective effects of carnosine in diabetic animal models could be related to a hypoglycemic effect, which is possibly a consequence of the ability of carnosine to regulate the autonomic nervous system or by effect, which is possibly a consequence of the ability of betic animal models could be related to a hypoglycemic effect. Such a mechanism was then further examined by Kurata et al. (226) demonstrating that even at much lower doses (intravenous injection 15 nmol/rat), carnosine efficiently prevented the I/R-induced renal injury and that a similar protective effect was achieved by an intracerebroventricular injection of the peptide (1.5–5 pmol/rat), thus suggesting its action on the central nervous system. Such histamine-mediated mechanism was supported by the lack of activity of N-acetylcarnosine, which is a carnosine derivative stable to carnosinas and by the suppression of the protective effect of carnosine by thioperamide which is a H3 receptor antagonist (226).

On the other hand, Shen et al. (338) demonstrated that the mechanism of carnosine action in a model of permanent middle cerebral artery occlusion may not be mediated by the histaminergic pathway. This pathway was also ruled out by Ma et al. (241) to explain the protective effect of carnosine in subcortical ischemic vascular dementia induced by carotid arteries occlusion. Carnosine (200, 500 mg/kg) but not l-histidine was found to ameliorate white matter lesions and cognitive impairment in both wild-type mice and histidine decarboxylase knockout mice (241). More recently, Wang et al. (386) showed that carnosine influences the mRNA expression of the signal transducer and activator of transcription 3 (STAT3) which would explain the inhibition of neuronal apoptosis after acute ischemic stroke.

D. Cancer

Carnosine has generated particular interest for its antiproliferative properties. As pointed out by Gaunitz and Hipkiss in a perspective on carnosine and cancer (144), the first report on the antineoplastic effect of carnosine was described in 1986 by Nagai and Suda (263), who reported that carnosine treatment (50 mg·kg⁻¹·day⁻¹) inhibited tumor growth and mortality in mice. Ten years later, Holliday and McFarland (187) reported that human fibroblasts grew normally in the presence of carnosine while it selectively inhibited the growth of neoplastic cell lines. A first hypothesis on the mechanism of action was then proposed, which is based on the ability of carnosine to deplete glycolysis...
intermediates (glyceraldehydes phosphate and dihydroxyacetone phosphate) by its carbonyl quenching ability and therefore to reduce the generation of ATP. Such a mechanism well agrees with the metabolic differences between these two cell types (Warburg effect) and in particular that most tumor cells are predominantly glycolytic for ATP supply, whereas in differentiated fibroblasts, ATP synthesis is predominantly aerobic and mitochondrial in origin. Cartwright et al. (79) showed that when yeast cells depend on glycolysis for energy generation, l-carnosine slows growth rates and increases cell death, but when they are reliant on oxidative phosphorylation, they become resistant to l-carnosine’s inhibitory effects.

More recently, the antiproliferative activity of carnosine was demonstrated in HCT16 colon cancer cells where carnosine was found to dose-dependently inhibit the ATP production (from 5 to 300 mM). In addition to targeting ATP, the authors also demonstrated the ability of carnosine to inhibit mitochondrial ROS generation, which is induced by KRAS oncogene and which is involved in cell proliferation (194).

The ability of carnosine to inhibit tumor growth in vivo was demonstrated by Renner et al. (314) in a nude mouse model in which NIH3T3 fibroblasts expressing the human epidermal growth factor receptor 2 were implanted in the dorsal skin. More recently, the ability of carnosine to inhibit tumor proliferation was confirmed in an animal model where human colon cancer (HCT116) cells were implanted in BALB/c athymic mice (188).

E. Aging

Carnosine is described as an anti-aging compound, affecting a wide range of age-related processes and functions. In 1994, McFarland and Holliday first reported the ability of carnosine to extend the maximum cell division capacity of cultured human fibroblasts converting senescent cells to juvenile phenotypes (252). Some years later, the same authors confirmed and extended such data, demonstrating that the addition of carnosine in the fibroblasts medium switched their phenotype from senescent to juvenile and that the effect was reversed by removing the peptide (253). The anti-aging effect of carnosine has also been described in animal models. The group of Boldyrev reported that carnosine supplemented to a standard diet attenuates the development of senile features and increases life span by 20% in senescence-accelerated mice (SAM) (68, 142, 402). Carnosine was also found to significantly increase the number of spermatogonia and Sertoli cells in mice prone to accelerated aging (149) and to increase the lifespan of male Drosophila flies (401). Although some have ascribed the anti-aging effect of carnosine to its antioxidant activity (26, 68), others proposed that the geroprotective effect of carnosine is mediated by its ability to prevent protein carbonylation and to modulate the degradation of aged proteins (175–177). Further studies are definitely required to investigate this important and popular potential application of carnosine, as well as the underlying mechanisms.

F. Neurological Disorders

The possible protective effect of carnosine in Alzheimer’s disease (AD) was first proposed in the late 1990s when in vitro studies demonstrated the ability of the dipeptide to inhibit, on one hand the formation of β-amyloid polymerization and on the other hand the cell toxicity. Preston et al. (306) reported that carnosine provided some protection against the effect of the neurotoxin β-amyloid peptide (Aβ25–35) on rat brain vascular endothelial cells. The cell protective effect of carnosine against amyloid-β (1–42) mediated toxicity was then confirmed in other cell models. Amyloid-β caused a dose-dependent loss of rat cerebellar granule cell neurons viability which was significantly inhibited by carnosine. The protective effect of carnosine was more recently demonstrated on amyloid-β (1–42) impairment of differentiated PC12 cells.

Only a few in vivo studies on the effect of carnosine in animal models of AD have thus far been reported. Corona et al. (89) studied the effects of carnosine treatment in triple-transgenic AD mice, who develop an age-related neurodegenerative phenotype that is driven by intraneuronal deposition of amyloid-β and accumulation of h-tau. It was found that carnosine had a strong effect in restoring mitochondrial functioning and in counteracting amyloid-β aggregation but had no effect in tau pathology and only a trend toward the amelioration of cognitive deficits (89). More recently, Herculano et al. (169) reported a prevention of cognitive decline by carnosine supplementation in transgenic AD mice fed a high-fat diet. Some supportive evidence in humans comes from a metabolomic study, consisting of screening the changes in free amino acid and dipeptide concentrations in AD patients compared with controls. It was found that carnosine levels were significantly lower in the plasma of AD patients with respect to controls (134).

Regarding the mechanism of the therapeutic action of carnosine in AD, the carnosine-histidine-histamine pathway was ruled out by using histamine receptor antagonists and histidine decarboxylase inhibitors. Likewise, the antioxidant activity of carnosine is unlikely as amyloid-β induced cell damage is not induced by generation of ROS. Other hypotheses were proposed, considering the antiglycating activity (63), the involvement in the regulation of glutamate release and NMDA receptor trafficking (137), and the transient and weak interactions between carnosine and the charged groups surrounding the central hydrophobic cluster of amyloid-β (1–42) thus inhibiting the self-assembly (25). In vitro and in vivo studies also indicate that a possible mechanism of neuroprotection of carnosine is mediated by
its metal ion chelating activity. The rationale for addressing Zn\(^{2+}\) dyshomeostasis in AD is substantial since Zn\(^{2+}\) may induce aggregation and oligomerization of amyloid-\(\beta\) and also cell toxicity. By using suitable fluorescent probes, Coron et al. (89) demonstrated the ability of carnosine to chelate Zn\(^{2+}\) in cultured glia cells. The cytoprotective effect was then supported by Horning et al. (190), demonstrating that physiologically relevant concentrations of carnosine provide a protective effect against toxicity induced by zinc and copper in cultured neurons.

In relation to Parkinson’s disease, carnosine was found to inhibit oligomerization of \(\alpha\)-synuclein in model systems (208, 214), and to suppress some changes in animal models of Parkinson’s disease (374). In a small clinical trial, Boldyrev et al. (62) found that carnosine treatment (1.5 g/day) in combination with L-DOPA therapy in Parkinson’s disease patients significantly improved a number of neurological symptoms, including decreased rigidity of the hands and legs, and increased hand movement and leg agility. At a biochemical level, a decrease in protein carbonyls in blood plasma and an increase of SOD have been found (62).

Another small clinical trial on the effect of carnosine on neurological disorder is a double-blinded placebo-controlled study on the effect of carnosine treatment (800 mg daily for 8 wk) on the observable changes in 31 children with autistic spectrum disorders. After 8 wk of carnosine but not placebo treatment, children showed statistically significant improvements on the Gilliam Autism Rating Scale and the Receptive One-Word Picture Vocabulary test (84). Interestingly, a recent metabolomic study revealed decreased levels of urinary carnosine, \(\beta\)-alanine, and \(L\)-histidine in children with autistic spectrum disorder (257). Unfortunately, no further clinical trials on this aspect have been reported, nor recorded in the database at www.clinicaltrials.gov.

Recently, the effect of carnosine treatment (2 g daily for 3 mo) in adults with chronic schizophrenia was studied in a randomized, double-blind, placebo-controlled trial (83). Carnosine but not placebo was found to improve some cognitive tests such as the strategic target detection test. The authors concluded that carnosine merits further consideration as adjunctive treatment to improve executive dysfunction in persons with schizophrenia (83). A mild positive effect of carnosine treatment (1.5 g daily for 12 wk) on cognitive function has been also reported in a small randomized double-blind placebo controlled trial involving involving Persian Gulf war veterans affected by cognitive dysfunction termed Gulf War illness (GWI) (45).

**G. Ocular Diseases**

Carnosine has been reported to positively affect some age- and diabetes-related ocular diseases. Most of the data are reported on cataract, and more specifically on the insoluble aggregates (predominantly \(\alpha\)-crystallin) which induce lens opacity. In vitro studies reported the ability of carnosine to inhibit \(\alpha\)-crystallin aggregation induced by methylglyoxal as well as to disaggregate glycated \(\alpha\)-crystallin. The latter effect referred to the ability of carnosine to increase peptide chain mobility resulting in an unfolding of the glycated protein (332). The ability of carnosine to modulate the aggregation-disaggregation dynamics was then confirmed and extended by Attanasio et al. (24), demonstrating the prevention of fibril formation of \(\alpha\)-crystallin alone or in a more complex lens model. Moreover, they found a disassembling effect of carnosine on \(\alpha\)-crystallin amyloid fibrils. The ability of carnosine to prevent cataract formation and development was evaluated in different animal models, and some controversial results were reported. Babizhayev (28) first reported that carnosine significantly prevented cataracts induced in the rabbit eye by the administration of lipid peroxidation products. In contrast, carnosine was found ineffective in preventing cataracts in streptozotocin-induced diabetic rats (234) or in an in vivo model of cataracts induced by sodium selenite (239). More recently, carnosine was found to delay the progression of lens opacification in diabetic rats, but only during the earlier stages of the disease (396). Clinical studies have been reported on the effect of N-acetylcarnosine on cataracts. Topical eye application of N-acetylcarnosine reportedly leads to a better accumulation of carnosine in the aqueous humor compared with a direct application of carnosine (32). In volunteers with senile cataracts, N-acetylcarnosine-treated eyes demonstrated visual improvement in general (30) and in particular an improvement in visual acuity and glare sensitivity (29, 31).

**H. Wound Healing**

Since the beginning of the 1980s, the enhancing effect of \(L\)-histidine and carnosine on wound healing was observed in animal models (130). The mechanism is probably related to the activity of both constitutive amino acids: \(L\)-histidine and \(\beta\)-alanine. On one hand, carnosine acts as \(L\)-histidine precursor, thus regulating histamine synthesis during trauma (130). Faster wound healing through histamine is associated with the activity of basic fibroblast growth factor, leading to macrophage recruitment and angiogenesis (276). On the other hand, the enhancement by carnosine of wound healing may also be ascribed to the stimulation of collagen biosynthesis by \(\beta\)-alanine (264). The efficacy of carnosine on wound healing was demonstrated in several animal models. Perel’sman et al. (296) established that carnosine was able to double the rate of wound reparative processes in the injured lung of guinea pigs, by activating fibroblast proliferation, connective tissue generation, and intracellular regeneration. More recently, carnosine has been shown to accelerate healing of bleomycin-induced and irradiation-induced pulmonary wounds (92, 155).
A large body of evidence exists on the healing effect of Polaprezinc (a zinc-carnosine complex, see sect. III) in gastric ulcers, an effect which is partially mediated by the stimulating effect of the metal complex on the production of insulin-like growth factor I (211, 222, 273, 333, 388). More recently, the effect of carnosine on wound healing was demonstrated in db/db mice, a model for type 2 diabetes. Carnosine treatment (100 mg/kg) significantly improved wound healing, and tissue analysis revealed higher expression of cytokine genes and growth factors involved in wound healing (17).

VII. CARnosinase AND DISEASE

In this section we discuss some diseases that have been linked to the carnosinase enzyme. This implies that their pathological features are in some way influenced by a dysregulation of carnosine or homocarnosine degradation.

A. CNDP1 Genotype and Disease Risk

Several genome-wide linkage scans for diabetic nephropathy have mapped susceptibility loci to chromosome 18q22.3-23 (73, 379), a large region which also includes the carnosinase genes CNDP1 and CNDP2. In 2005, Janssen et al. (197) managed to further fine map this region and provided the first evidence for a genetic link between serum carnosinase (CNDP1) and diabetic nephropathy. A trinucleotide repeat in exon 2 of CNDP1 determines the number of leucine residues (5, 6 or 7) in the leader peptide of the CN1 precursor. European diabetic patients homozygous for the five leucine allele were less susceptible to diabetic nephropathy and had lower serum carnosinase activity. A later study on Cos-7 transfected cells (315) clarified that the five leucine alleles impair the efficiency of secretion of serum carnosinase, which may explain why serum carnosinase activity is linked to CNDP1 genotype. The disease-protecting effect of lower serum carnosinase activity probably relates to higher stability of carnosine in the circulation (128) or alternatively to a local effect of enhanced carnosine availability in the kidney (221). The current working hypothesis for the link between genetic predisposition and pathology is shown in Figure 16.

The link between the (CTG)\textsubscript{n} polymorphism of the CNDP1 gene and diabetic nephropathy was confirmed by an independent study on European Americans with type 2 diabetes (136), but could not be found in patients with diabetic nephropathy due to type 1 diabetes (387) nor in African-Americans (251). Mooyaart et al. (260) demonstrated that the protection against development of diabetic nephropathy is only present in women, but not in men with the 5–5 leucine polymorphism. Recently, other single-nucleotide polymorphisms (SNPs) in the CNDP1 and CNDP2 genes have also been associated with diabetic nephropathy (7, 225, 251).

![Figure 16. Proposed pathogenetic link between the leucine-repeat variation in the CNDP1 gene and the predisposition to develop diabetic nephropathy.](http://physrev.physiology.org/)
The accumulation itself of the carnosine and homocarnosine in the brain is indicative of a malfunction of the dipeptide hydrolysis enzyme. Later studies have confirmed the absence or low activity of serum carnosinase (131, 297). When a meat-free diet is ingested, carnosinemia and carnosinuria are suppressed but nonetheless still present, in contrast to healthy individuals (392).

Reported symptoms of patients with serum carnosinase deficiency are mental retardation, hypotonia, myoclonic seizures, and spasticity, which usually manifest in the first year of life (298, 377, 392). However, it appears that the severity of symptoms is not related to the residual activity of serum carnosinase (88), and serum carnosinase deficiency can occur in the absence of neurological symptoms. Dietary meat restriction will partially reduce carnosinemia, but will not relieve clinical symptoms.

Homocarnosinosis has been described in only one Norwegian family and is characterized by elevated concentrations of homocarnosine in brain and CSF (147, 299). The mother as well as the two sons and one daughter all show these metabolic changes, whereas only the children have neurological symptoms: motor dysfunction, retinal pigmentation, and mental deterioration. Similar to patients with serum carnosinase deficiency, the homocarnosinosis patients have carnosinuria and reduced capacity to hydrolyze dietary HCD. Chronic dietary restriction of l-histidine, which is one of the precursors of (homo-)carnosine, did markedly reduce CSF homocarnosine concentration and carnosinuria, but did not relieve the symptoms (240).

Initially, it was proposed that homocarnosinosis patients lack homocarnosinase activity in the brain in contrast to healthy individuals (299). However, Lenney et al. (231) indicated that homocarnosinase was not present in normal human brain tissue either. The ability of brain extracts to hydrolyze homocarnosine therefore appeared to be attributable to the carnosinase that is still present in the serum trapped in a brain sample. As patients with homocarnosinosis have extremely low serum carnosinase activity, it appears that homocarnosinosis is in fact also attributable to serum carnosinase deficiency (231). Despite this probably common biochemical cause, there remain some dissimilarities between homocarnosinosis and serum carnosinase deficiency, e.g., with respect to the age of onset of symptoms and the type of symptoms.

Our current knowledge fails to understand a possible link between serum carnosinase on the one hand and the neurological symptoms on the other hand. It seems likely that the accumulation itself of the carnosine and homocarnosine in various body compartments is not posing the problem. They are not to be considered as neurotoxins at high concentrations. Therefore, it is more likely that their functional utilization, possibly as a source of GABA, β-alanine, or l-histidine production, is causing the symptoms in affected patients. A better insight in the pathogenesis in these patients would allow us to better understand the normal physiological role of the (homo-)carnosine/carnosinase system.

The scientific literature on the genetic basis of carnosinase deficiency is still very scarce at present. The activity of serum carnosinase is usually suppressed in parents and siblings of patients and shows the pattern of an autosomal recessive disorder. Willi et al. (389) reported the case of a 30-mo-old girl with serum carnosinase deficiency. The long arm of her paternal chromosome 18 had a terminal deletion at breakpoint q21.3. The father had normal but the mother had reduced serum carnosinase activity. Therefore, the patient was likely hemizygous for the defect, having received the deficiency allele from her mother and, as a result of a de novo generated deletion, no allele from her father (389). Another recent progress on this topic was made by Zschocke et al. (409), who identified a null mutation in the CNDP1 gene. This newly discovered frameshift mutation (L17fsX20) would introduce a stop codon at position 36 of the amino acid chain of CN1. Individuals homozygous for L17fsX20 are predicted to have a complete absence of serum carnosinase activity, and therefore carnosinemia. The null mutation was found to have a carrier frequency of 1.4% in a German population, and the prevalence for individuals with homozygosity for L17fsX20 is on this basis estimated to be 1:20,000 (409). No such patients have yet been identified.

In addition to the above-described inherited metabolic disorders, there are other diseases characterized by lowered serum carnosinase activity. Young patients with progressive muscular dystrophy or myopathy and patients with hypothyroidism have been shown to have very low serum carnosinase activity (41, 43). Whether this enzymatic abnormality is causally related or just coincidental to the condition is not known. Because carnosinase is a serum enzyme that is mainly produced and secreted by the liver, it is not surprising that patients with chronic liver disorders also display markedly reduced serum carnosinase activity (42), attributable to low serum CN1 content (300).

**C. The Human Serum Carnosinase Paradox**

An unbalanced activity of serum carnosinase (CN1), either too high (increased risk of diabetic nephropathy) or too low (carnosinase deficiency), is apparently leading to diseased state. These clinical cues may help us to decipher the enigmatic role of serum carnosinase. One could think that the role of serum carnosinase is to regulate the serum carnosine concentration (i.e., keep it within a certain homeostatic range). However, this is somewhat unlikely. First, a large number of mammals, including carnivores and omnivores...
A. Determinants of Muscle Carnosine Content

There is a considerable variation in muscle carnosine content among humans, as three- to fourfold differences have been demonstrated between the lowest (~10 mmol/kg dry wt) and highest (~40 mmol/kg dry wt) reported levels in humans (126, 163, 355). Despite the large interindividual variation, the intraindividual variation is rather limited. Repeated measurements of muscle carnosine over time in the same person displays only small fluctuations (39), and very high correlations have been found for muscle carnosine content within monozygotic twin pairs (35). Below, we describe the most important determinants that are responsible for the remarkable interindividual variation in muscle carnosine concentrations.

1. Fiber type

There is a clear difference in the carnosine content of different fiber types. It has repeatedly been reported that human fast-twitch (type II, white) fibers contain 30–100% more carnosine compared with slow-twitch (type I, red) fibers, based on HPLC analysis of pooled single fibers (162, 171, 213, 356). Similar fiber type differences have been documented in a wide range of mammals (116, 117, 336, 337). Within the human population, individuals that have fast-twitch fibers as the predominant fiber type in their muscles will have higher muscle carnosine levels compared with those who mainly possess slow-twitch fibers (245, 336, 349). Examples of the former type of people are athletes who excel in sprint-type and high-intensity exercise, such as 100-m runners, short-track skaters, etc., whereas examples of the latter include endurance-type athletes such as marathon runners, triathletes, etc. Baguet et al. (37) have used a noninvasive technique based on proton magnetic resonance spectroscopy (1H-MRS) to quantify muscle carnosine content in a diverse group of elite athletes. They observed markedly higher carnosine content in the sprinters compared with the endurance athletes, in agreement with previous reports using biopsies (289). Baguet et al. (37) thus proposed the use of 1H-MRS as a noninvasive estimation method for muscle fiber type composition.

2. Sex

Both biopsy (243) and MRS (126) studies have shown that men display considerably (22–82%) higher carnosine levels compared with women. At prepubertal age however, this sexual dimorphism is not yet present (35) (see Figure 17). These findings suggest that androgens have a positive effect on the muscle carnosine synthesis and content. Bodybuilders, likely using anabolic steroids, have indeed been shown to hold very high muscle carnosine concentrations (355). An animal study by Penafiel et al. (294) provided supportive data for this, as they observed that castration of mice re-
duced muscle carnosine levels by 40%, while testosterone administration to female mice increased muscle carnosine content by 268%. A similar study on orchidectomy with or without testosterone replacement in mice confirmed these observations and clarified that the mechanism of testosterone’s effect is not based on enhanced transcription of the carnosine synthase enzyme but more likely through the increased expression of the taurine/β-alanine transporter (125).

3. Age

There are no longitudinal data available, but cross-sectional studies demonstrate that muscle carnosine levels are negatively correlated with age (104, 200, 346, 356). In a study on 263 subjects of ages 9–83 yr, Baguet et al. (35) reported that the lower carnosine content at old age is mainly the result of a decrease that occurs during adulthood, even shortly after puberty, rather than a decrease from adulthood to elderly (see FIGURE 17). The cause for the decline in muscle carnosine with advancing age is unclear, but a decline in androgens/estrogens and fiber type changes may be contributing factors.

4. Other

It has been suggested that exercise training can also alter muscle carnosine content (350). However, most short-term training intervention studies seem to agree that a few weeks of training will not stimulate carnosine accumulation in muscle (36, 212, 213, 244). It remains to be established whether long-term (months to years) training intervention is able to modulate muscle carnosine content, especially when this chronic exercise intervention is possibly accompanied by a shift in fiber type composition (327). Other determinants of muscle carnosine content, such as diet, which will be discussed in the following part.

B. Dietary Manipulation of Muscle Carnosine Content

The synthesis rate of carnosine in muscle cells appears to be rate-limited by the availability of β-alanine, rather than L-histidine (118). L-Histidine, an essential amino acid, is present in the blood in sufficient concentrations and will probably only impose a limitation to muscle carnosine synthesis in the case of a L-histidine-free diet (357). The endogenous supply of β-alanine is presumably dependent on the hepatic synthesis from uracil degradation. However, this metabolic route is poorly understood and possibly inadequate for the total β-alanine demand. Therefore, exogenous and thus dietary supply of β-alanine seems to be of relevance as well. The average daily intake of β-alanine from an omnivore Western diet has been calculated to amount to ~330 mg/day (126), nearly all of which is derived from animal products (red meat, white meat, fish, etc.) in the form of HCD.

As indicated in section II, there is a large variation in muscle HCD content between animal species. Therefore, the choice of animal-derived dietary ingredients will markedly determine the daily HCD intake (166). Examples of food sources in the human diet specifically rich in HCD are chicken, turkey, tuna, horse, and whale, containing the equivalent of 2–7 g β-alanine per kg ww. Beef, pork, deer and rabbit meat have more intermediate concentrations (1–2 g/kg ww β-alanine). Some fishes, such as mackerel and sole, contain virtually no HCD. As there exist large differences in HCD content between muscle types within a species, there is also large variability between the various meat cuts, which can easily differ by a factor of 3 or more, in favor of the white meat portions (containing more type II fibers and thus HCD) (3, 261). Although carnosine has a good chemical stability, small losses may occur in the HCD content of the meat during the production process. Cooking of beef muscle will decrease muscle carnosine content by 5–20% (308). Dry-curing of ham from pork biceps femoris muscle will decrease HCD content by 35% over a 10-mo period (249), but these losses are less pronounced than those for creatine.

Dairy and vegetable food products do not contain HCD nor β-alanine, except from the reported presence of β-alanine in vegetable oils, albeit in concentrations 100- to 1,000-fold lower than in meat (323). As shown in FIGURE 18, vegetarians, almost exclusively relying on endogenous sources of β-alanine, have been shown to possess >20% lower muscle carnosine content compared with omnivores (126). This confirms the initial hypothesis that endogenous β-alanine production is probably insufficient for unrestricted muscle carnosine synthesis. Only short-term intervention studies (5 wk) are available at present and do not seem to indicate a significant reduction in muscle carnosine content when transiently switching omnivores onto a vegetarian diet (36). However, the latter finding probably is caused by the long half-life of carnosine in tissues. Possibly longer duration

![FIGURE 17](image-url) Carmosine content in the gastrocnemius medialis muscle of healthy men (n = 271) and women (n = 217) of different ages, measured by NMR spectroscopy (data compiled from Refs. 35, 126). Each symbol represents the average of 10 healthy subjects, ranked by age.
vegetarianism is required to longitudinally demonstrate a decline in muscle carnosine content. On the other hand, raising the dietary intake of β-alanine has been shown to be an effective and powerful means to induce muscle carnosine loading (166).

The regular ingestion of a chicken breast extract (CBEX), which is high in HCD content (325) and is a popular dietary product in Asia, can elevate muscle carnosine content in rats (258, 351). In 2006, Harris et al. (165) have shown that the daily ingestion of a very high dose of l-carnosine (13 g/day) continued for 4 wk could elevate muscle carnosine content of healthy volunteers by 65%. Interestingly, a similar increase was obtained when ingesting pure β-alanine, rather than carnosine, in an isomolar dose, which lends further credit to the conclusion that (dietary) β-alanine is rate limiting for muscle carnosine content (165). A number of independent groups (39, 102, 343) have since confirmed this finding of muscle carnosine loading through chronic β-alanine supplementation (1.6–6.4 g/day for several weeks) and have further optimized this procedure [reviewed in Harris et al. (166) and Stellingwerff et al. (344)]. The muscle carnosine loading effect of chronic β-alanine supplementation has also been shown in mice (127), although much higher doses are required to obtain the same effects (FIGURE 18).

So far, the highest reported increases in human muscle carnosine content are 80–85% upon 10–12 wk of supplementation (102, 171). Stellingwerff et al. (344) indicated that the major determinant of supplementation-induced carnosine loading is the total ingested dose over a supplementation period, rather than the daily amount. Coingestion of β-alanine supplements with meals seems to promote carnosine synthesis, which points to a possible role of insulin in this process (342). Once accumulated in the muscle, the elevated carnosine content remains present for an extended period upon cessation of supplementation. The effective washout period (return to presupplementation baseline) apparently amounts to 10–20 wk (39, 344). Little side-effects have been reported for β-alanine ingestion in the concerned doses, except from transient and unpleasant itching/flushing sensations on the skin, called parasthesia (165). To circumvent this, slow-release β-alanine tablets have been developed which result in slower absorption kinetics and which reduce the incidence of parasthesia to the level of placebo (101). The mechanism of the itching/tingling sensation upon oral β-alanine has recently been ascribed to Mas-related G protein-coupled receptor member D (MrgprD), a G protein-coupled receptor, expressed in dorsal root ganglion neurons involved in cutaneous mechanosensation (236).

Several questions with regard to dietary manipulation of muscle carnosine content remain to be answered. It still has to be determined whether or not a normal (omnivore) variation in the type and amount of meat ingestion will influence the muscle carnosine concentration between individuals. An initial comparison of high versus low meat eaters did not show a difference in muscle carnosine content (126). Furthermore, the upper safe limit of muscle carnosine loading is still unknown. Finally, it is unclear whether dietary intake of β-alanine has also consequences for HCD content of nonmuscle tissues. In this respect, Tomonaga et al. (367) recently showed that acute CBEX administration in rats can elevate the HCD concentrations in certain brain regions.

C. Ergonomic Potential of Carnosine Loading

The above-described β-alanine supplementation-induced muscle carnosine loading strategy has been shown to be beneficial for certain types of exercise performance. In 2007, Hill et al. (172) reported that the capacity to cycle at
110% of maximal power ($W_{\text{max}}$) was improved by 13 and 16% following, respectively, 4 and 10 wk of chronic β-alanine supplementation in healthy untrained volunteers. Later studies confirmed that ergogenic effects can also be obtained in well-trained athletes, such as cyclists and rowers (34, 378). However, not all studies have been able to demonstrate beneficial effects of carnosine loading on exercise performance (105, 212), which probably relates to the type of exercise. In a recent meta-analysis based on 15 experimental studies on β-alanine and exercise performance, Hobson et al. (185) came to the conclusion that β-alanine is beneficial in exercise types lasting 1–4 min, whereas the effect was less pronounced ($P = 0.046$) in longer duration exercises (>4 min) and was nonsignificant for shorter duration exercise (<1 min). More well-designed studies are definitely needed to better define the precise performance-enhancing effects of carnosine loading. In the meanwhile, β-alanine is rapidly gaining world-wide popularity as an ergogenic nutritional supplement in the sports community.

D. Application in Food Science

There are many applications of the measurement of the concentration of carnosine and other HCD to gain information about the composition of food products. For example, carnosine and anserine seem to be missing from several industrially designed chicken soups, which indicates that no chicken meat is used for its production (170). Another example is the analysis of HCD to detect products of animal origin in feed for ruminants, which is often forbidden, because of the increased risk of bovine spongiform encephalopathy (BSE) (328). Also, the source of meat (chicken, pork, beef) can be traced by the ratio of carnosine to anserine in a meat product (365).

Human urinary concentrations of carnosine, anserine, and especially Nπ-methyl-histidine (the degradation product of anserine) have been proposed to be good biomarkers of dietary meat intake, which can be used as a compliance tool in dietary intervention studies (111) or as an estimate in epidemiological studies investigating the effects of meat intake on health and disease (90).

Given the multitude of potential health-enhancing effects, carnosine has been identified as one of the promising bioactives in meat (19). Several approaches can be developed to take advantage of this characteristic. The functional or enriched meat approach aims to increase the total HCD content of meat. Different dietary strategies, such as carnosine and l-histidine supplementation (168, 220, 242), are currently explored in farm animals to increase the quality of the meat as well as the HCD content, with potential health benefit for the consumer. Alternatively, extraction procedures may be applied to meat products to obtain a HCD-rich extract, such as chicken essence, as a functional food (232). Clearly, the potential applications of HCD in the field of meat and food science continue to evolve.

IX. CONCLUSIONS AND FUTURE PERSPECTIVES

A. Why Did the Carnosine System Evolve?

The above sections have summarized a large body of literature on the biochemical, physiological, and therapeutic properties of carnosine. A complex and energy-consuming molecular system has been established in the course of evolution to synthesize, modulate, transport, and degrade carnosine across the organism. In this section, we now aim to combine these individual findings into a global vision on the role of HCD in the function of organisms. A number of questions can be asked related to why this system evolved and why it evolved in the way it did: Why carnosine and not free l-histidine? Why does l-histidine need to be combined with β-alanine, and not with another amino acid? Why the methylated carnosine analogs?

To resolve these questions, it is necessary to compare and summarize the biochemical properties of carnosine with its constituent amino acids, and with the other HCD in one table (TABLE 2). From TABLE 2, it can be deduced that most of the bioactive functions of carnosine relate to histidine and its imidazole moiety, except from its role in AGEs and ALEs formation and calcium regulation, which is accomplished much better by the dipeptide. From this information, we propose the structure-function relationship of carnosine, depicted in FIGURE 19, which will be used as a basis for the discussion below. The methylated carnosine analogs are mostly similar in functionality compared with carnosine, although there are some slight differences in effectiveness.

1. Why carnosine and not free l-histidine?

From the comparison of the biochemical properties of carnosine versus l-histidine (TABLE 2), it can be deduced that most of the functional properties of carnosine are related to the l-histidine part (mostly the imidazole moiety), of which the dipeptide is composed. In other words, as roughly only 1 of 20 amino acids is l-histidine in proteins, HCD are a way to more effectively store histidine in peptide form. Yet, it can be questioned why nature did bother to create an ATP-consuming carnosine synthesis system, if nearly the same roles can be accomplished by the simple free l-histidine itself. In fact, it seems that having free l-histidine, instead of the HCD, is probably a good solution to a certain extent, concerning most of the homeostatic challenges for which an organism is in need of the imidazole-related properties. As described in section II, several families of fishes have a low concentration of HCD, but instead high millimolar concentrations of l-histidine, whereas mammals and other animal classes with high HCD content, will only have a micromolar concentration of free l-histidine. Thus free l-histidine was a reasonably good solution earlier in verte-
brate evolution, but the system has further evolved, became clearly more complex, and improved. So what are the additional advantages of a L-histidine-containing dipeptide over free L-histidine?

A first probable reason is improvement and better suitability to the functions. As an example, the $pK_a$ value of the imidazole of free L-histidine is 6.0. This is close to the physiological pH range, but not exactly in it. By combining L-histidine with $\beta$-alanine, the $pK_a$ of the imidazole alters to 6.72, which is simply a better version of the buffer (even though the pH buffering property in carnosine is still attributable to the L-histidine part). Similar fine-tuning can be observed for other biochemical/physiological properties as well (TABLE 2). More importantly, some functions, such as the inhibiting effect of the formation of ALEs (see sect. III), seem to be specific to the dipeptide, and can poorly be replicated by L-histidine alone.

A second probable reason is guaranteeing a stable tissue concentration. L-Histidine is involved in many pathways, such as protein synthesis and histamine formation; pathways with high physiological priority. To avoid highly fluctuating tissue L-histidine content, it has probably proven beneficial to capture L-histidine into a metabolically more inert dipeptide. Even though there is a system to degrade carnosine (see sect. IV on carnosinases), the hydrolyzing enzyme is usually not present in the compartments where carnosine is synthesized and subsequently stored.

A third possible reason may relate to reduced toxicity. It has been suggested that dipeptides are less toxic than single amino acids, for example, to neurons in the brain. Also, single amino acids probably more easily initiate and activate unwanted metabolic pathways than dipeptides.

2. Why does L-histidine need to be combined with $\beta$-alanine?

It seems that in the “choice” for an ideal amino acid to bind with L-histidine in the dipeptide, preference is given to a rare and nonproteinogenic amino acid ($\beta$-alanine). A first advantage of this is to stabilize the dipeptide by reducing the affinity towards hydrolysis by (di)peptidases. If L-histidine would be bound to another proteinogenic amino acid, it would be readily hydrolyzed by the peptidases. However, carnosine is not at all a good substrate to peptidases, which thereby allows a separately regulated and specific metabolism (carnosinase) compared with other dipeptides. Not only for hydrolysis, but also for synthesis, choosing a rare (beta) amino acid that is not involved in many more path-

<table>
<thead>
<tr>
<th>Activity</th>
<th>Carnosine</th>
<th>Anserine</th>
<th>Homocarnosine</th>
<th>Ophidine</th>
<th>$\beta$-Alanine</th>
<th>Histidine</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant (scavenging effect)</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>/</td>
<td>**</td>
<td>100, 209, 219</td>
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<tr>
<td>Peroxy radicals</td>
<td>**</td>
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<td>/</td>
<td>**</td>
<td>69, 85, 209</td>
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<td>Hydroxyl radicals</td>
<td>**</td>
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<td>**</td>
<td>ND</td>
<td>/</td>
<td>**</td>
<td>133</td>
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<tr>
<td>Peroxynitrite</td>
<td>**</td>
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<td>ND</td>
<td>ND</td>
<td>/</td>
<td>**</td>
<td>121</td>
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<tr>
<td>Singlet oxygen</td>
<td>**</td>
<td>**</td>
<td>ND</td>
<td>ND</td>
<td>*</td>
<td>*</td>
<td>271</td>
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<tr>
<td>Nitric oxide</td>
<td>**</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>/</td>
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<td>3</td>
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<tr>
<td>Buffering activity</td>
<td>$pK_a$</td>
<td>**</td>
<td>ND</td>
<td>**</td>
<td>ND</td>
<td>*</td>
<td>382</td>
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<tr>
<td>Metal ion chelating</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>**</td>
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<td>Cu$^{2+}$ chelation</td>
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<td>ND</td>
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<td>AGEs inhibition</td>
<td>**</td>
<td>**</td>
<td>ND</td>
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<td>*</td>
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<td>transglycation</td>
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<td>**</td>
<td>ND</td>
<td>ND</td>
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<td>*</td>
<td>312</td>
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<td>ND</td>
<td>ND</td>
<td>*</td>
<td>*</td>
<td>9</td>
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</tbody>
</table>
| Marks summarize the activity of carnosine derivatives and of the constituent amino acids in respect to carnosine: **, equal; *, lower: ***, greater; /, no activity; ND, not determined.

FIGURE 19. Structure-activity relationship of carnosine. The imidazolic ring is required for the antioxidant, metal ion chelating and buffering activity. The $\beta$-alanine moiety regulates the metabolic fate of the L-histidine residue and synthesis of carnosine. Both $\beta$-alanine and L-histidine residues synergistically act in the inhibiting effect towards AGEs and ALEs formation.
ways like protein synthesis seems more easy to control. Indeed, \(\beta\)-alanine is clearly the rate-limiting precursor in the synthesis of carnosine. So to regulate the carnosine concentration, it is sufficient to regulate the availability of \(\beta\)-alanine. It has recently been proposed that there is a specific enzyme in muscle, glutamate decarboxylase-like protein 1 (GADL1), directly synthesizing \(\beta\)-alanine from aspartate by decarboxylation (235), which could be a demonstration that \(\beta\)-alanine availability is controlled by a specific and unique set of pathways to indirectly regulate tissue carnosine content.

3. Why the methylated carnosine analogs?

The distribution of HCD in muscles from different species throughout the animal kingdom, as described in section II, may help us to understand the reason and advantage for having the methylated versus the nonmethylated carnosine and for having one methylated form over the other (anserine versus ophidine). It is very likely that these typical patterns found in animal families are not a mere coincidence and have a specific reason. As indicated in Table 2, the differences in the chemical properties between carnosine, anserine, and ophidine are only minor. However, there are slight differences in the pH buffering capacity (\(pK_a\) is 6.72 for carnosine, 7.01 for anserine, and 7.03 for ophidine), and also the antioxidant capacity. When certain animals predominantly express one type of HCD, this could mean that the chemical property, for which this HCD is optimal, is the primary reason to have this HCD present in the organism.

However, it seems more likely that the reason for carnosine methylation is related to the fact that anserine and ophidine are less easily hydrolyzed by carnosinase than carnosine itself. The methylation of carnosine could therefore be a way to protect HCD from hydrolysis and be a better way to store and ascertain constant HCD concentrations in certain cells and tissues. Yet why do humans, as the unique species of all mammals (see FIGURE 3), only have carnosine and not the methylated forms? One reason could be that the protection against carnosinase-induced hydrolysis through carnosine methylation is not required in humans. Even the contrary, the absence of methylated carnosine analogs in combination with the high expression of serum carnosinase in humans compared with other animals even suggests that the HCD pool in human tissues is less stable and should be more mobile to contribute to other physiological processes. This hypothesis, however, needs further testing.

B. Future Perspectives

Although the research interest in carnosine is now clearly gaining momentum (106), we are currently still missing the critical mass needed to systematically resolve the remaining missing links in the carnosine system. For this purpose, an informal Carnosine Consortium (http://users.unimi.it/carnosine_co/) was recently established, with the objective to organize regular international meetings on this topic and to facilitate international collaboration. Some enzymes (e.g., mammalian CMT) still need to be discovered, transgenic animals are waiting to be developed (e.g., knockout of CS), and systematic efforts in the fields of genetics, pharmacology, etc. would greatly enhance the required scientific progress in the field.

Carnosine has pleiotropic effects. This is probably one of the reasons why this system evolved in the first place. One molecule can fix several problems: a broad-spectrum solution. At the same time, this pleiotropy imposes great challenges to the researchers. Most studies have been focusing only on a single aspect of the molecule, thereby overlooking the other aspects. This should be taken into account in the design of future research.

Carnosine is a promising therapeutic molecule. A human-specific problem is the rapid degradation upon oral or intravenous administration by serum carnosinase. The development of carnosinase-resistant carnosine analogs is therefore a major challenge, but a sure hurdle that needs to be taken to allow future pharmaceutical progress. In addition, a better understanding of diseases that suffer from reduced tissue carnosine contents, and of the mechanisms regulating tissue concentration, may offer therapeutic perspectives as well.

Over 100 years past the discovery of carnosine, we are still no where near a good and complete understanding of its physiological and pathophysiological significance, but we hope that this review has helped the reader to grasp the current status on the scientific knowledge of this intriguing molecule.

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During the period of writing and preparing this manuscript, one of the authors, Alexander Boldyrev, passed away. We honor Professor Boldyrev with this review paper as a tribute to the immense contribution he has made to the field of carnosine research. The assistance of Audrey Baguet in the preparation of the manuscript is greatly acknowledged.

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