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STUDIES ON HOMOCARNOSINOSIS AND ON HUMAN TISSUE CARNOSINASE AND ITS INHIBITION BY BESTATIN AND BY ENDOGENOUS INHIBITORS

University of Hawaii

Ph.D. 1984

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STUDIES ON HOMOCARNOSINOSIS AND ON HUMAN TISSUE CARNOSINASE
AND ITS INHIBITION BY BESTATIN AND BY ENDOGENOUS INHIBITORS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOMEDICAL SCIENCES
(PHARMACOLOGY)
DECEMBER 1984

by

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enzymes. I also want to thank John L. Reardon and Janet Shin for their technical assistance in some of the assays.

To my wife, Maureen, I want to express my gratitude for her understanding, patience and great encouragement. Finally, I would also like to thank my parents for their financial assistance and encouragement during my graduate education.
ABSTRACT

Carnosine (β-alanyl-L-histidine) is a major constituent of human skeletal muscle. Its function in muscle is yet undetermined, but there is evidence that carnosine may serve as a buffer, an activator of myosin ATPase, or a source of supplementary histidine or histamine. Carnosine is also highly localized in the olfactory bulb of the mouse, where it is a putative neurotransmitter for olfactory chemoreceptor neurons. Tissue carnosinase may be responsible for its degradation and consequent inactivation at the synapse. Another histidine dipeptide, homocarnosine (γ-aminobutyryl-L-histidine), is almost exclusively localized in the CNS; little is yet understood about its physiological function.

Patients afflicted with homocarnosinosis have elevated concentrations of homocarnosine in brain and cerebrospinal fluid. It has been reported that they lack brain homocarnosinase. However, in the present study it was found that these patients are deficient in serum carnosinase, a dipeptidase (mol.wt. 160,000) which hydrolyzes homocarnosine in addition to carnosine. When an extract of normal human brain was chromatographed on DEAE-cellulose, all homocarnosine-hydrolyzing activity was present in one peak and had a molecular weight of 160,000. Also, homocarnosinase (Mol.wt. 57,000) was not detected in brain extracts after isoelectric focusing. Homocarnosine-hydrolyzing activities
and serum carnosinase activities of human CSF samples were significantly correlated (p<0.001). In homocarnosinosis patients, both of these activities were negligible in serum samples, a CSF sample, and a brain biopsy sample when compared to corresponding controls. Thus, the ability of brain extracts and CSF to hydrolyze homocarnosine appears to be attributable solely to serum carnosinase, which is deficient in patients with homocarnosinosis. The homocarnosine-hydrolyzing activity in 12 other tissues was related to the amount of trapped blood, but brain contained 15 times the activity expected from its blood content; hence, serum carnosinase may be synthesized in the brain.

Human tissue carnosinase was found to be optimally active at pH 9.5. It was inhibited by p-hydroxymercuribenzoate and activated by 2 mM dithiothreitol, indicating it to be a cysteine peptidase. By optimizing assay conditions, tissue carnosinase activities per g of tissue were 5- to 15-fold greater than those previously reported. The enzyme was present in every human tissue assayed and was entirely different from plasma carnosinase. Highly purified tissue carnosinase had a broader specificity than hog kidney carnosinase. Although tissue carnosinase was very strongly inhibited by bestatin, it did not hydrolyze tripeptides and thus appears to be a dipeptidase rather than an aminopeptidase. It was found to have a molecular weight of 90,000, an isoelectric point of 5.6, and a $K_m$ value of 10 mM
carnosine under the conditions of assay. Two forms of kidney and brain carnosinase were separated by high resolution anion exchange chromatography, although only one form was detected using various electrophoretic methods. Two other enzymes, homocarnosinase and manganese-independent carnosinase, were not detected in human tissues, but were readily measured in rat and hog kidney.

Crude extracts of human tissues contain endogenous tissue carnosinase inhibitors that were dialyzable and thermostable over a pH range of 1 to 11. Among 14 different tissues the amount of inhibition varied considerably, but no tissue was without inhibitor. An endogenous inhibitor from human liver was isolated and identified as L-leucine. It was found to be the most inhibitory \((IC_{50} = 0.2 \text{ mM})\) of all the common amino acids, followed by cysteine and cystine. L-leucine inhibition was stereospecific, D-leucine being inactive, and was partially dependent on manganese; this indicates that it binds to the active site of tissue carnosinase. In contrast, cysteine probably inhibited carnosinase by chelating manganese; as manganese concentrations were increased above 0.02 mM, the inhibition decreased. Cystine inhibition was attributed to its reduction to cysteine by the dithiothreitol present in the digest. Since the concentrations of leucine and other amino acids in a liver extract accounted for only one fourth of its inhibitory activity, another inhibitor(s) must have been
present. Inhibition by L-leu-L-leu was greater than by L-leucine, and plots of the reciprocal velocity vs. L-leucine concentration displayed upward curvature; these results suggest that L-leucine binds to two sites on tissue carnosinase. L-leucine inhibition was mixed competitive and noncompetitive, being predominantly competitive at concentrations below 0.2 mM L-leucine and more noncompetitive at higher concentrations.

Bestatin is a dipeptide that has potential clinical value as an immunostimulant and chemotherapeutic agent. Commonly known to inhibit leucine aminopeptidase and aminopeptidase B, bestatin was found to be an extremely potent inhibitor of human tissue carnosinase ($K_i = 0.5$ nM). The affinity of bestatin binding to tissue carnosinase was 18 to 40 times greater than to nonparticulate leucine aminopeptidase and 120 times greater than to aminopeptidase B. Bestatin was a competitive inhibitor and was most inhibitory at a manganese concentration close to the optimum for enzyme activity. The structures of bestatin and carnosine are similar; both contain an N-terminal $\beta$-amino acid connected to an $\alpha$-amino acid having an R-group with a branch-point at the $\gamma$-carbon. Therefore, it is proposed that bestatin binds with high affinity to tissue carnosinase because its backbone chain is identical to that of carnosine.
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CHAPTER I
INTRODUCTION

Carnosine (β-alanyl-L-histidine) was first isolated from beef muscle by Gulewitsch and Amiradzibi (1900) and its structure was determined by Baumann and Ingvaldsen (1918) and by Barger and Tutin (1918). It has long been recognized that carnosine and anserine (β-alanyl-L-methylhistidine) are the most abundant compounds next to creatine in the non-protein nitrogenous fraction of vertebrate muscle (du Vigneaud and Behrens 1939). They may comprise as much as 3% of the dry weight of some muscles (Crush, 1970). The amount of each imidazole dipeptide is known to vary considerably between species, muscle tissues and individuals of the same species (Wood, 1957; Crush, 1970; Christman, 1976; Tamaki et al., 1976; Seely and Marshall, 1981; Abe, 1983). Recently, Carnegie and coworkers (1983) found that the total amount of imidazole dipeptides in meat samples from 10 different mammals is surprisingly similar (averaging about 16 μmoles/wet g), even though the concentration of each of the peptides varies greatly from species to species. Interestingly, the ontogenetic appearance of these dipeptides coincides with the onset of mechanical functioning of the muscle (Skvortsova, 1953). Human skeletal muscle contains between 1.0 and 8.3 μmoles of carnosine per wet g but lacks anserine (Perry et al., 1967; Christman,
1971, 1976). In contrast, the muscles of monkeys (Macacus rhesus) contain as much as 23.4 μmoles/g of carnosine and 0.49 μmoles/g of anserine (Christman, 1976).

The biological function of carnosine in muscle remains to be established. Deutsch and Eggleton (1938), finding that the $pK_a^2$ values of carnosine and anserine are 6.9 and 7.1, respectively, have proposed that these dipeptides may be significant buffers in the physiological pH range. In some muscles, 20-30% of the total buffering capacity could be attributed to carnosine and anserine (Bate-Smith, 1938). Muscles having high concentrations of imidazole dipeptides tend to be those which derive much of their metabolic energy anaerobically and are poorly vascularized (Zapp and Wilson, 1938; Davey, 1960b; Lykkeboe and Johansen, 1975). Carnosine and anserine may therefore play an important role in neutralizing the lactic acid produced in skeletal muscles.

Carnosine also activates myosin ATPase. Bowen (1965) has shown that ATP-induced contraction of glycerol-treated muscle fibers is accelerated by carnosine. Activation of myofibrillar-ATPase occurs in the absence of added calcium (Yun and Parker, 1965) and appears to be significant (66% activation) at physiological concentrations (10 mM) (Avena and Bowen, 1969). Carnosine has been shown to activate myofibrillar-ATPase from carnosine-containing muscles of vertebrates but not from the invertebrate muscles having little or no carnosine or anserine (Parker and Ring, 1970).
Thus, the effect may be limited to myofibrils from muscles that normally contain one or both dipeptides as major constituents. A fraction of the total carnosine in muscle may be bound to myofibrils; repeated washing of myofibrils with 50% ethanol or 50% glycerol have failed to liberate most of this "bound" carnosine (Reddy and Hegsted, 1962). Severin et al. (1979) have shown in autoradiographs a bound pool of carnosine localized in the interfibrillar space corresponding to the cisternae; however, it is not known whether this represents soluble carnosine trapped in glutaraldehyde-fixed membranes or covalently bound carnosine.

Carnosine is a well known chelator of heavy metals (Rosenberg, 1960d; Brown and Antholine 1979b, 1980; Brown, 1981; Brown et al., 1982) and the activation of several enzymes by carnosine has been correlated to the removal of toxic metals (Dobbie and Kermack 1955; Davey, 1960a; Ikeda et al., 1980a&b). It is possible that the inhibition of dopamine-β-hydroxylase (Izumi et al., 1975) and the activation of muscle phosphorylase (Johnson et al., 1982; Johnson and Aldstadt, 1984) by carnosine under in vitro conditions may also be attributable to the buffering or chelating effects of carnosine.

Carnosine in muscle may, at particular times, be a supplementary source of histidine for the body. Du Vineaud et al. (1937) have shown that dietary carnosine may be
utilized for growth in place of histidine. Muscle carnosine concentrations drop rapidly when young chickens or rats are fed histidine-free diets (Ousterhout, 1960; Quinn and Fisher, 1977b) or a diet containing 50% of the histidine required for growth (Barbaro et al., 1978). In contrast, anserine concentration is not affected by dietary histidine restriction. Carnosine in muscle could also be hydrolyzed to produce histidine and/or histamine during times of trauma (Fisher et al., 1978), inflammation (Horisaka and Hashimoto, 1979), infection (Tsuchiya et al., 1970; Fitzpatrick et al., 1980) or wound healing (Nagai, 1975; Vizioli and Almeida, 1978; Fitzpatrick and Fisher, 1982).

In addition to muscle, nervous tissue of mammals also contains imidazole dipeptides (Hosein and Smart, 1960; Pisano et al., 1961; Abraham et al., 1962; Dickinson and Hamilton, 1966; Kanazawa and Sano, 1967; van Balgooy and Roberts, 1975; Grove et al., 1982b). Regional distribution studies of mammalian brains have revealed much higher concentrations of carnosine present in the olfactory bulb and epithelium (about 2 μmole/g) than in other areas (Margolis, 1974, 1981; Neidle and Kandera, 1974; Ferriero and Margolis, 1975; Easter and Baker, 1977; Quinn and Fisher, 1977a; Wideman et al., 1978).

There is considerable evidence that carnosine may be a neurotransmitter in the olfactory pathway of the mouse brain (for reviews, see Margolis 1978, 1980, 1981). Many of the
biochemical criteria essential for establishing a compound as a neurotransmitter have been met. The only major requirement yet to be satisfied is that postsynaptic application of carnosine mimics the electrophysiological response to stimulation of olfactory neurons, or that carnosine plays a physiological role in olfaction. Preliminary studies of the electrophysiological response of olfactory bulb neurons to carnosine have not been conclusive (Tonosaki and Shibuya, 1979; MacLeod, 1978; MacLeod and Strughan, 1979; Nicoll et al., 1980; Gonzalez-Estrada and Freeman, 1980). Also, Quinn and Fisher (1977a) did not detect any loss in olfactory ability of rats that had been fed a histidine-free diet to lower the olfactory bulb carnosine concentration.

Carnosine is localized in the olfactory chemoreceptor neurons. When the primary afferent neurons of the olfactory epithelium are selectively destroyed by intranasal irrigation with zinc sulfate, causing degeneration of their axons and synaptic terminals in the olfactory bulb, the carnosine concentration in olfactory bulbs drops by 90%, whereas most other amino compounds are unaffected (Margolis, 1974). The results match the localization and behavior of the unique marker protein of the primary olfactory pathway after the same treatment (Margolis et al., 1974). Central denervation by bulbectomy produces a similar pattern of carnosine loss in the olfactory mucosa, reportedly due to
retrograde degeneration of the primary olfactory receptor neurons (Ferriero and Margolis, 1975; Margolis et al., 1976). Olfactory bulb carnosine is localized predominantly in the fiber and glomerular layers, the locations of olfactory nerve axons and terminals, respectively (Nadi et al., 1980).

Carnosine is synthesized in the primary olfactory neurons from its constituent amino acids by a specific enzyme. Neidle and Randera (1974) have demonstrated the in vitro conversion of histidine into carnosine in olfactory bulb extracts. Intranasal irrigation with [14C]histidine or [14C]α-alanine results in the synthesis of [14C]carnosine, some of which is transported by axoplasmic flow to the olfactory bulb (Harding and Margolis, 1976; Margolis and Grillo, 1977). The radiolabeled carnosine accumulates mostly in the glomerular layer of the olfactory bulb, consonant with the postulation of its neurotransmitter role (Burd et al., 1982).

Carnosine synthetase from rat brain has been partially purified and found to require ATP, Mg$^{2+}$ and NAD (Skaper et al., 1973). More recently, Horinishi and coworkers (1978) were able to purify 600-fold and characterize the carnosine synthetase from the mouse olfactory bulb. This enzyme is also strictly dependent on ATP and Mg$^{2+}$, but has no dependence on NAD. The mouse olfactory bulb enzyme has a high substrate specificity for L-histidine and α-alanine,
but is also capable of incorporating γ-aminobutyric acid (GABA) into homocarnosine (γ-aminobutyryl-L-histidine). Carnosine synthetase is thought to be responsible for carnosine and homocarnosine synthesis in the olfactory pathway as well as in other regions of the central nervous system (Kish et al., 1979). Within the olfactory bulb and epethelium, carnosine synthetase appears to be localized in the receptor neurons; axotomy results in almost complete loss of the enzyme activity in both tissues (Harding et al., 1977). The synthetase has been found in the cytosolic fraction of mouse olfactory tissue, with little activity in the synaptosomal fraction (Harding and O'Fallon 1979). The enzyme is reportedly more concentrated in neuronal than glial cell-enriched fractions of whole brain (Ng et al., 1977). However, Bauer and coworkers (1979, 1982a&b) have demonstrated that glioma cells and glial cells in primary culture are also able to synthesize and release carnosine. In view of the dissimilar properties exhibited by the preparations of carnosine synthetase from rat whole brain (Skaper et al., 1973) and from mouse olfactory bulb (Horinishi et al., 1978), perhaps there are two isozymes of the synthetase; one isozyme may be highly localized in neurones, while the other may be mostly in glial cells. This would be analogous to the enzymes responsible for acetylcholine hydrolysis; acetylcholinesterase occurs exclusively in the neurons, whereas nonspecific
cholinesterase is present in glial cells (Guroff, 1980). That there may be two carnosine synthetases is also suggested by the presence of two activity peaks in a Sephadex G-100 chromatograph of a rat brain extract (Skaper et al., 1973). Unfortunately, the apparent molecular weight of either peak has not been reported, and its comparison with the synthetase from mouse olfactory bulb is not possible.

Carnosine is released from olfactory bulb synaptosomes by two mechanisms (Rochel and Margolis, 1982). The first is a fast release mechanism dependent upon membrane depolarization and stimulated by calcium. The other is a slower, spontaneous process independent of depolarization or calcium.

Membrane fractions from mouse olfactory bulb also exhibit saturable, reversible, and stereospecific binding sites for carnosine, with a $K_d$ of about 770 nM (Hirsch et al., 1978). Deafferentation or treatment of the olfactory bulb membranes with trypsin greatly decreases the number of binding sites (Hirsch and Margolis, 1979). Within the olfactory bulb the density of carnosine binding sites is greatest in the glomerular layer (Nadi et al., 1980).

The olfactory pathway also contains a specific degradative enzyme, carnosinase (Harding and Margolis, 1976; Margolis et al., 1979). Since a high-affinity uptake system for carnosine in synaptosomal membrane fractions has not been found (Rochel and Margolis, 1982), there is a strong
possibility that carnosinase may act as an inactivating system in olfactory neurons. This would be analogous to the enzymatic degradation of other neurotransmitters, e.g., acetylcholine and enkephalins. In the olfactory epithelium, hydrolysis of carnosine appears to occur extraneuronally; axotomy produces only a minor reduction of carnosinase activity there (Harding and Margolis, 1976; Harding et al., 1977). But in the olfactory bulb, the hydrolysis of carnosine is reduced to 40% of the control following axotomy (Harding et al., 1977); this suggests that the olfactory bulb carnosinase is at least partially associated with terminals of chemoreceptive neurons. Margolis et al. (1979) have presented conclusive evidence that the predominant form of carnosinase in mouse olfactory mucosa, kidney and uterus is a separate enzyme from the predominant carnosinase found in the central nervous system, including the olfactory bulb. The mouse kidney carnosinase was purified by Margolis et al. (1983), who found that it does not require exogenous Mn$^{2+}$ for activity. It was thereby referred to as the Mn-independent carnosinase to distinguish it from the other enzyme, the Mn-dependent carnosinase, which was not purified (Margolis and Grillo, 1984). A study of the immunocytochemical localization of Mn-independent carnosinase showed that within the olfactory mucosa only the glandular cells are immunoreactive, suggesting that the enzyme has a secretory function (Margolis et al., 1983). The main olfactory bulb shows no immunoreactivity to this carnosinase.
A detailed study of the olfactory bulb carnosinase, requiring exogenous Mn$^{2+}$ for activation, has not yet been published. Although the hydrolytic activity against carnosine in olfactory bulb was reported to be only 4% of that in the olfactory epithelium (Harding and Margolis, 1976), the assays were performed under suboptimal conditions for activity of the bulb enzyme (Lenney et al., unpublished). In view of the localization of the other carnosinase in glandular cells, this Mn-dependent carnosinase is more likely to be involved in regulating the hydrolysis of synaptically released carnosine. Major portions of this dissertation will describe the partial purification and characterization of Mn-dependent carnosinase from human brain and other organs (Chapter III), the identification and study of its endogenous inhibitors (Chapter IV), and an investigation into its inhibition by a tight-binding inhibitor, bestatin (Chapter V).

**Homocarnosine** (GABA-histidine) is another imidazole dipeptide present in mammalian brain (Pisano et al., 1961). Among mammals, homocarnosine is almost exclusively localized in the central nervous system and occurs in greater amounts in human brain than in any other mammalian brain (Abraham et al., 1962). The dipeptide is one of the major amino compounds of human brain, where it is found at higher
concentrations than carnosine in all regions (Abraham et al., 1962; Kanazawa and Sano, 1967; Perry et al., 1968; Gjessing and Sjaastad, 1974).

Since this dipeptide is a conjugate of GABA, homocarnosine may serve as a reservoir for this central nervous system inhibitory neurotransmitter. Studies using GABA-transaminase inhibitors to elevate brain GABA levels have clearly demonstrated the ability of GABA to be incorporated into homocarnosine in vivo (Bohlen et al., 1980b; Grove et al., 1981; Tell et al., 1981). GABA can also be released from homocarnosine in human brain (Perry et al., 1979b) and cerebrospinal fluid (Grove et al., 1982a&b). (The enzyme responsible for brain and CSF homocarnosine hydrolysis is identified in Chapter II.)

The interconversion of homocarnosine and GABA is not proof that these compounds have closely related physiological functions. If homocarnosine serves as a storage form of GABA, one might expect these compounds to have parallel distribution patterns in different brain areas. Studies of autopsied human brain regions (Abraham et al., 1962; Kanazawa and Sano, 1967; Perry et al., 1971a; Young and Snyder, 1973; Kish et al., 1979) have not shown a consistent correlation between the levels of homocarnosine and GABA. However, Perry et al. (1971b, 1981) have demonstrated that these compounds undergo major concentration changes shortly after death; their levels in autopsied brain do not
accurately represent the amounts during life. Biopsied samples of human brain regions appear to have dissimilar distribution patterns for homocarnosine and GABA (Perry et al., 1971b). Furthermore, the concentrations of homocarnosine during rat brain development follow a very different pattern than GABA (Tiemeyer et al., 1982). Enwonwu and Worthington (1973, 1974) reported that some brain regions of malnourished monkeys showed profound increases of homocarnosine (as great as 2.5-fold) compared to adequately fed monkeys; the GABA contents of these regions were not significantly altered. Therefore, it appears that homocarnosine has a function that is not directly connected with the role of GABA.

Homocarnosine has been reported to have a stabilizing influence on neurons; introduction of homocarnosine into the CSF in dogs inhibited seizures induced by electrical stimulation (Hayashi, 1966). However, others have noted correlations that indicate the opposite. For example, CSF homocarnosine levels were higher in uncontrolled epileptic children than in controlled epileptic children, although both groups were treated with similar medication (Takahashi, 1981). Control of infantile spasms with ACTH was associated with a decreased CSF homocarnosine concentration (Ohtsuka et al., 1983). Furthermore, elevated CSF homocarnosine levels have been implicated in the mediation of dyskinetic movements by treated Parkinsonism patients (Robin and
There is no solid evidence at the present time that clearly shows the physiological role homocarnosine plays in the brain.

Perry et al. (1968b) have shown that normal children have much higher CSF levels of homocarnosine than normal adults. Also, phenylketonuria patients who have not undergone therapy have higher CSF homocarnosine concentrations than do patients who have been treated (van Sande et al., 1970). Noting that high CSF homocarnosine is associated with both undeveloped and degenerate brains, conditions favoring a high glial cell/nerve terminal ratio, Fonnum (1984) has proposed that glial cells may be responsible for synthesis and/or storage of homocarnosine. Indeed, glial cells in culture from mouse brain are capable of homocarnosine synthesis (Bauer et al., 1982). However, carnosine synthetase, which is able to synthesize homocarnosine, was reported to be localized also in mouse olfactory pathway neurons (Harding et al., 1977) and in rat brain neuron-enriched fractions (Ng et al., 1979).

Abnormal concentrations of homocarnosine in the CSF or in regions of the brain have been reported with several neurological diseases, including Huntington's chorea (Perry et al., 1973; Bohlen et al., 1980a), schizophrenia (Perry et al., 1979a), phenylketonuria (van Sande et al., 1970) and homocarnosinosis (Gjessing and Sjaastad, 1974). Homocarnosinosis patients have greatly elevated CSF and brain
concentrations of homocarnosine (Gjessing and Sjaastad, 1974; Perry et al., 1979) and are afflicted with a progressive paraplegia, mental deterioration, and retinal pigmentation. The next chapter (II) describes the biochemical defect responsible for the abnormal homocarnosine levels in these patients.

The purposes of this research are two-fold. The primary purpose is to investigate the characteristics of human tissue carnosinase and its inhibitors. Although a Mn-dependent hog kidney carnosinase (Hanson and Smith, 1949; Rosenberg, 1960a,b,&c) and a Mn-independent mouse kidney carnosinase (Margolis et al., 1983) have been thoroughly studied, the human tissue carnosinase is distinctly different and has never been characterized. Human tissue carnosinase is Mn-dependent and seems to be homologous to the mouse olfactory bulb Mn-dependent carnosinase; thus, tissue carnosinase may be responsible for degradation of synaptically released carnosine in the olfactory pathway. Tissue carnosinase may also be a pharmacological receptor for bestatin, a potentially important immunostimulant and chemotherapeutic agent; therefore, the characteristics of this enzyme inhibition by bestatin are examined in this thesis. Because endogenous tissue carnosinase inhibitors may be involved in regulating its activity in vivo, the tissue distribution of these inhibitors was examined and an inhibitor was isolated, identified and studied.
The second purpose of this research is to establish whether serum carnosinase is the only human enzyme to split homocarnosine. Patients afflicted with homocarnosinosis are deficient in homocarnosine-splitting activity attributed to homocarnosinase (Perry et al., 1979b), but the results reported herein demonstrate the deficient enzyme to be serum carnosinase.
CHAPTER II

SERUM CARNOSINASE DEFICIENCY ASSOCIATED WITH HOMOCARNOSINOSIS

INTRODUCTION

Homocarnosinosis is a rare metabolic disorder which has been reported in three members of one Norwegian family by Gjessing and Sjaastad (1974) and by Sjaastad et al. (1976, 1977). These patients have CSF concentrations of homocarnosine (GABA-histidine) approximately 20 times the normal value (Sjaastad, 1974) and are afflicted with spastic paraplegia, progressive intellectual impairment, and retinal pigmentation. Sjaastad (1981) describes the dominant symptom as a progressive spasticity that is at first restricted to lower extremities, but eventually leads to paraplegia in extension. The progressive mental deterioration correlates with a marked cortical cerebral atrophy in the proband which had been studied by a brain biopsy. The third characteristic feature is a grey, diffuse, bilateral macula area pigmentation of the retina, but with the patients retaining normal visual acuity (Sjaastad, 1981).

A hog kidney dipeptidase (homocarnosinase) which hydrolyzes homocarnosine was described by Lenney et al. (1977). This enzyme was present in rat uterus, kidney, and
liver but was not detectable in rat serum or brain using a sensitive fluorometric assay. Kish et al. (1979) reported that homocarnosinase was present in human brain. Perry et al. (1979) analyzed a frontal cortex biopsy sample from one of the homocarnosinosis patients and found it contained four times the normal concentration of homocarnosine, and was lacking homocarnosinase. The high concentrations of homocarnosine in cerebral cortex and CSF was attributed to the deficiency in brain homocarnosinase.

Lunde and coworkers (1982) studied the compounds excreted by the homocarnosinosis patients when they consumed a meal high in anserine and carnosine. As compared with normal controls, the patients excreted higher levels of carnosine and anserine and lower concentrations of histidine and 1-methylhistidine.

Lenney et al. (1982) found that human serum carnosinase differed from human tissue carnosinase. The circulating enzyme hydrolyzed homocarnosine, whereas the tissue dipeptidase did not.

In this section, it is shown that all homocarnosine-hydrolyzing activity of normal human brain and CSF is attributable to serum carnosinase, and that homocarnosinosis patients are deficient in this enzyme; the enzyme homocarnosinase was not detected. Furthermore, samples of normal human brain contained much more homocarnosine-hydrolyzing activity than expected on the basis of the
content of trapped blood. The possibility that serum carnosinase is synthesized in the brain is considered.

MATERIALS AND METHODS

Materials

Serum samples from the three homocarnosinosis patients, a cerebrospinal fluid (CSF) sample from one of these patients, and a cerebral cortex biopsy sample from another of these patients were shipped by air on dry ice from Norway to Honolulu, along with serum samples from three normal volunteers and from the parents of the homocarnosinosis patients and two cerebral cortex biopsy samples from control patients. Serum samples were also obtained from employees of Queens Medical Center and from certain in-patients and out-patients. All control CSF samples were from in-patients at Queens Medical Center.

The sources of equipment and supplies were as follows:
model 200 ultrafiltration cell and Diaflo YM30 ultrafiltration membranes, Amicon (Danvers, MA);
homocarnosine, Aldrich (Milwaukee, WI); DEAE-cellulose, Bio-Rad Labs (Richmond, CA); charcoal (Norit A), J.T. Baker (Philipsburg, NJ); disc gel electrophoresis chemicals, Eastman Kodak (Rochester, NY); ampholytes, Isolab (Akron, OH); all other chemicals (including carnosine, Sephadex, aldolase, bovine serum albumin, and ovalbumin), Sigma
Enzyme assays

To measure the activity of tissue carnosinase, the following solutions were sequentially measured into borosilicate (12 x 75 mm) digest and blank tubes using positive displacement pipettes: 0.20 ml of 125 mM sarcosine-HCl buffer (pH 9.5), 0.10 ml of 10 mM dithiothreitol, and 0.10 ml of an appropriately diluted tissue carnosinase solution containing 0.25 mM MnCl₂. After 15 min for activation at 22°C, the reaction was initiated by adding 0.10 ml of 100 mM carnosine, pH 9.5, to the digest tubes. Digest and blank tubes were incubated at 30°C for 30 min, and then 0.50 ml of 0.6 N trichloroacetic acid was added to each tube and 0.10 ml of substrate was added to the blank tubes.

The quantity of histidine released by the enzyme was measured fluorometrically by a modification of the method of Ambrose et al. (1969). To 0.05 ml of the trichloroacetic acid supernatant (10 min at 800 x g) was added 1.20 ml of 0.83 N NaOH and 0.05 ml of 2% o-phthalaldehyde in methanol. After 15 min at 30°C, 0.50 ml of 4 M phosphoric acid was added and the mixture was incubated for 15 min at 30°C and for 60 min at 22°C. The tubes were protected from direct sunlight, which enhances fluorescence. Readings were taken using a Perkin-Elmer Model 650-10M fluorescence
spectrophotometer (excitation at 340 nm and emission at 440 nm) standardized with a tetraphenylbutadiene solid standard (Perkin-Elmer #4).

Differences in fluorescence between the digest tubes and their corresponding blanks were converted into umoles of histidine by reference to a histidine calibration curve prepared from various mixtures of histidine, \( \beta \)-alanine and carnosine representing 0 to 20\% hydrolysis. Histidine alone cannot be used to calibrate the method because carnosine has a significant quenching effect on the fluorescence obtained from histidine.

Serum carnosinase was measured using a similar procedure (Lenney et al., 1982), except that the digest tubes contained 20 mM carnosine, 0.5 mM CdCl\(_2\), 3 mM sodium citrate, and 50 mM NH\(_4\)OH-HCl buffer, pH 8.5.

In measuring homocarnosine-hydrolyzing activity, the digest contained 4 mM homocarnosine, 0.4 mM CoS\(_4\), and 50 mM Tris-HCl buffer, pH 7.6. Sensitivity was increased by the use of a micro modification of the histidine assay. To 0.2 ml of the trichloroacetic acid supernatant were added 0.3 ml of 1.0 M NaOH, 0.2 ml of 0.2\% o-phthalaldehyde in 95\% ethanol, and 0.2 ml of 4M H\(_3\)PO\(_4\). Other details of the procedure were as described above.

Normal human tissues for the homocarnosine-hydrolyzing assay were obtained at autopsy, which was performed 5-20 h after death. The organs or tissue samples were placed on Chemical Co. (St. Louis, MO).
ice for about 1 h and then they were frozen at -70°C. Excess blood had drained off during the period before freezing. To 5 g of frozen tissue was added 25 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM MnCl₂ and 0.02% NaN₃. This suspension was homogenized for 1 min at 22°C in a Virtis homogenizer and clarified by centrifugation. The supernatant was dialyzed overnight at 4°C with stirring against 900 ml of 1 mM MnCl₂ containing 5 g of charcoal.

**Column chromatography**

Human cerebral cortex (25 g) was homogenized at 22°C with 50 ml of 5 mM Tris-HCl buffer, pH 8.0. The homogenate was centrifuged and the pellet was extracted with 50 ml of the same buffer. The combined extracts (71 ml) was applied to a 2 x 44 cm bed of DEAE-cellulose which had been equilibrated with the Tris buffer. The column was washed with this buffer until the absorbance of the eluate at 280 nm dropped below 0.05. An 800-ml linear NaCl gradient (0-0.5 M) in the same buffer was then passed through the bed. The procedure was conducted at 22°C and the eluate was collected in 12.5-ml fractions.

The molecular weights of enzymes were estimated using the procedure of Whitaker (1963). A 1.5 x 112 cm bed of Sephadex G-200 was eluted with 10 mM phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.02% NaN₃. Calibrating proteins were aldolase (MW=160,000), bovine serum albumin (MW=67,000), and ovalbumin (MW=45,000).
Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out according to Davis (1964) using a stacking gel, but no sample gel. Tissue (2 g) was homogenized with 3 ml of H$_2$O; the extract was dialyzed overnight at 4°C against 0.1 mM MnCl$_2$. After electrophoresis of the extract (25 μl) at 8°C for 2 h at 30 mA in the presence of 0.1 mM MnCl$_2$, the gel rod was cut into 5-mm sections. To each finely diced section was added 0.1 ml of each of the following: 0.125 mM MnCl$_2$, 10 mM dithiothreitol, 250 mM sarcosine-HCl buffer (pH 9.5), and 100 mM carnosine. The suspensions were incubated with occasional shaking for 2 h at 30°C and then overnight at 4°C. Then 0.5 ml of 0.6 N trichloroacetic acid was added and the tubes were centrifuged. A portion (0.2 ml) of the supernatant was analyzed for histidine by the method described above.

Isoelectric focusing in a sucrose gradient was conducted according to Behnke et al. (1979). A brain extract was prepared as for polyacrylamide gel electrophoresis; 2 ml were used in a 4-ml sucrose gradient containing 2.5% ampholyte, pH 3-10.
RESULTS

Serum carnosinase assays

Serum samples from the homocarnosinosis patients, their parents, control patients, healthy adults, and elderly out-patients were analyzed for serum carnosinase activity and homocarnosine-hydrolyzing activity (Table I). The control samples were within the normal range, whereas the patient samples displayed little or no activity against carnosine or homocarnosine. Serum carnosinase was undetectable in the mother of the homocarnosinosis patients and, for the father, was at the low end of the range for elderly out-patients.

Optimum conditions for homocarnosine hydrolysis

Crude brain extracts, serum samples, and partially purified serum carnosinase (Lenney et al., 1982) were all analyzed under various conditions to ascertain the optimum conditions for hydrolysis of homocarnosine. The three preparations were indistinguishable in regard to the parameters investigated. Tris-HCl, N-ethylmorpholine-HCl and NH OH-HCl buffers were compared, and Tris-HCl gave the highest activity. Among the metals tested (Ca$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Co$^{2+}$ and Zn$^{2+}$), cobalt was the most effective activator. The optimum pH of 7.6 and a $K_m$ value of 0.4 mM homocarnosine was obtained for all three preparations.
TABLE I

ACTIVITY OF SERA FROM HOMOCARNOSINOSIS AND CONTROL PATIENTS AGAINST CARNOSINE AND HOMOCARNOSINE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex</th>
<th>Age</th>
<th>Carnosine (μmoles/ml/h)</th>
<th>Homocarnosine (μmoles/ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>M</td>
<td>42</td>
<td>0.3 (5)</td>
<td>0.10 (2)</td>
</tr>
<tr>
<td>Patient B</td>
<td>M</td>
<td>38</td>
<td>0.1 (3)</td>
<td>&lt;0.05 (2)</td>
</tr>
<tr>
<td>Patient C</td>
<td>F</td>
<td>44</td>
<td>&lt;0.1 (2)</td>
<td>&lt;0.05 (1)</td>
</tr>
<tr>
<td>Mother of A, B, &amp; C</td>
<td>F</td>
<td>73</td>
<td>&lt;0.1 (1)</td>
<td>--</td>
</tr>
<tr>
<td>Father of A, B, &amp; C</td>
<td>M</td>
<td>76</td>
<td>11.0 (1)</td>
<td>--</td>
</tr>
<tr>
<td>Control 1</td>
<td>M</td>
<td>50</td>
<td>20.0 (1)</td>
<td>0.45 (1)</td>
</tr>
<tr>
<td>Control 2</td>
<td>F</td>
<td>49</td>
<td>23.0 (1)</td>
<td>0.50 (1)</td>
</tr>
<tr>
<td>Control 3</td>
<td>M</td>
<td>30</td>
<td>20.0 (1)</td>
<td>0.63 (1)</td>
</tr>
<tr>
<td>Healthy adults *</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=40)</td>
<td>M&amp;F</td>
<td>&gt;21</td>
<td>18-140</td>
<td>0.4-1.6</td>
</tr>
<tr>
<td>Elderly patients **</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=13)</td>
<td>M&amp;F</td>
<td>66-94</td>
<td>11-90</td>
<td>--</td>
</tr>
</tbody>
</table>

* Healthy adults were employees of Queens Medical Center.

** Elderly patients were out-patients of unknown medical condition.
Anion exchange chromatography of human brain extracts

An extract of normal human cerebral cortex was chromatographed on a DEAE-cellulose column at pH 8.0 as described in the "Methods" section. The effluent fractions were analyzed for ability to hydrolyze carnosine using the tissue carnosinase and serum carnosinase assay procedures and homocarnosine using its optimal conditions for hydrolysis. The results (Fig. 1) show that two peaks of activity were detected. Peak I fractions were more active in hydrolyzing carnosine under tissue carnosinase than serum carnosinase assay conditions and were unable to hydrolyze homocarnosine. Peak II fractions hydrolyzed both carnosine and homocarnosine. The ratio of carnosine hydrolysis under tissue carnosinase conditions to that under serum carnosinase conditions was much lower for peak II than for peak I fractions. No other peaks of activity against either of these substrates were detected in the fractions before the salt gradient or fractions eluting between 0.3 and 0.5 M NaCl. The experiment shown in Fig. 1 was repeated four times with a similar outcome each time.

The peak I fractions were pooled, concentrated by ultrafiltration on a YM30 membrane, and chromatographed on a calibrated Sephadex G-200 column. The peak II fractions were handled similarly. The peak I and II enzymes had apparent molecular weights of 90,000 and 160,000, respective-
Fig. 1. DEAE-cellulose column chromatography of a crude human cerebral cortex extract. Hydrolysis of carnosine was measured using the tissue carnosinase assay (○) and serum carnosinase assay (○) procedures. Hydrolysis of homocarnosine (●) and the estimated NaCl concentration (---) are also shown.
Carnosine hydrolyzed (μmoles/ml/h)

Homocarnosine hydrolyzed (μmoles/ml/h)

NaCl concentration (M)

Elution Volume (ml)
ly, and only the latter was active against homocarnosine.

Human tissue carnosinase from kidney and serum carnosinase were partially purified as described earlier (Lenney et al., 1982). When a mixture of these two preparations was chromatographed on DEAE-cellulose, the tissue carnosinase eluted early in the salt gradient and the serum carnosinase emerged thereafter.

**Homocarnosine-hydrolyzing activity in human tissues**

Thirteen human tissues were analyzed for their ability to hydrolyze homocarnosine. The hydrolysis rates were compared in Fig. 2 to literature values of the volume of blood in animal tissues after maximum free bleeding (Hansard and Friedman, 1971). The homocarnosine-hydrolyzing activity for most tissues was directly proportional to the percent of trapped blood. Lung and spleen were lower in activity than expected, perhaps because of differences in sample handling between the two studies. Brain (cerebral cortex) had an exceptionally high activity, much greater than predicted by the literature values of trapped blood. Several samples of human brain were then analyzed by the method of Dahlberg (1983) and found to contain about 1.5% blood. From Fig. 2, this correlates with a hydrolysis rate of about 0.015 umol/h. Thus, brain appears to contains about 15 times the homocarnosine-hydrolyzing activity which would be expected on the basis of its residual blood content.
Fig. 2. Relationship between homocarnosine-splitting activity of human tissues and the amount of residual blood in mammalian organs after maximum free bleeding. Tissues are: adrenal gland (A), brain (B), heart (H), small intestine (I), kidney (K), liver (Li), lung (Lu), skeletal muscle (M), pancreas (P), spleen (Sp), stomach (St), testis (T), and uterus (U). An ovary was also assayed (0.068 μmoles/g/h), but the blood content is unknown. Percent blood retained are average values from cattle, dog, rabbit, rat, sheep, and swine tissues (Altman and Dittmer, 1971).
Homocarnosine hydrolyzed (μmoles/g/h) vs Percent blood retained
Frozen brain biopsy samples from one of the homocarnosinosis patients (C) and two controls were received from Norway. These were analyzed along with a sample of autopsied brain for their abilities to hydrolyze homocarnosine. Hydrolysis of carnosine by the samples was also measured by the serum and tissue carnosinase assay procedures. The results are shown in Table II. The brain sample from the homocarnosinosis patient was unable to hydrolyze homocarnosine but hydrolyzed carnosine quite actively using the tissue carnosinase procedure. The small amount of activity via the serum carnosinase assay is attributed to tissue carnosinase. The brain (cerebral cortex) of this homocarnosinosis patient appears to be deficient in serum carnosinase but normal in the amount of tissue carnosinase.

**Serum carnosinase in CSF**

CSF samples from twelve control patients and from one of the homocarnosinosis patients (Patient A, Table I) were analyzed for activity against carnosine using the serum carnosinase assay procedure and for homocarnosine-hydrolyzing activity. When the data were plotted (Fig. 3) a significant linear correlation ($p < 0.001$) was observed between the two hydrolytic activities. Linear regression revealed that the homocarnosine-hydrolyzing activity averaged 1.6% of the rate of carnosine hydrolysis. Deviations from this proportion-
TABLE II
HOMOCARNOSINE AND CARNOSINE HYDROLYSIS BY BRAIN BIOPSY SAMPLES FROM A HOMOCARNOSINOSIS PATIENT AND CONTROL PATIENTS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Homocarnosine</th>
<th>Carnosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Tissue</td>
</tr>
<tr>
<td></td>
<td>carnosinase</td>
<td>carnosinase</td>
</tr>
<tr>
<td>Patient C</td>
<td>&lt; 0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>Control 4</td>
<td>0.18</td>
<td>6.5</td>
</tr>
<tr>
<td>Control 5</td>
<td>0.72</td>
<td>21.3</td>
</tr>
<tr>
<td>Control 6*</td>
<td>0.09</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Control 6 was an autopsied brain obtained 1 1/2 years prior to this assay. Other samples are biopsies obtained approximately 6 years prior to the assay. All samples were stored frozen until immediately prior to assay.
Fig. 3. Relationship between CSF hydrolytic activity against carnosine, as measured by the serum carnosinase assay, and homocarnosine-splitting activity. CSF samples are from control patients (O) and one of the homocarnosinosisis patients (●).
ality are most likely attributed to error in measuring the amount of homocarnosine hydrolyzed, which is near the limit of histidine detection. CSF from the homocarnosinosis patient did not hydrolyze carnosine and had negligible homocarnosine-hydrolyzing activity (Fig. 3). Serum carnosinase activity of CSF samples averaged about 10% of the activity of normal serum samples.

These CSF samples were also analyzed using the optimum conditions for tissue carnosinase; activity was only 4% of that measured using the serum carnosinase procedure.

Pooled samples of CSF were concentrated 5-fold by ultrafiltration on a YM30 membrane. The retentate was placed on a calibrated Sephadex G-200 column; the carnosinase had exactly the same elution volume as serum carnosinase (mol.wt. 160,000).

**Polyacrylamide disc gel electrophoresis**

An extract of human kidney was subjected to polyacrylamide disc gel electrophoresis. As shown in Fig. 4, two carnosinase peaks were detected; the larger one showed an R_f of 0.41 and the smaller one an R_f of 0.61. When extracts of brain or placenta were subjected to electrophoresis, two peaks of carnosinase activity similar to those in Fig. 4 were observed. In all cases, the smaller peak (R_f = 0.54-0.62) had a higher R_f than the larger peak (R_f = 0.38-0.45). No activity against homocarnosine was detectable after electrophoresis of brain, kidney, or placenta extracts.
Fig. 4. Polyacrylamide disc gel electrophoresis of a crude human kidney extract at pH 8.9 in a 7.5% gel. After electrophoresis, 5-mm sections of the gel rod were macerated and analyzed for activity vs. carnosine under optimum conditions for tissue carnosinase.
**Isoelectric focusing**

When a crude cerebral cortex extract was subjected to isoelectric focusing in a sucrose gradient, tissue carnosinase focused at pH 5.6. Carnosine hydrolysis measured under serum carnosinase assay conditions did not show an additional peak; serum carnosinase is unstable at its isoelectric point (Lenney et al., 1982). No homocarnosine-hydrolyzing activity was detectable.

**DISCUSSION**

When normal autopsied brain extracts were chromatographed on DEAE-cellulose (Fig. 1), only one peak of homocarnosine hydrolysis was detectable and corresponded to the second of two carnosine-hydrolyzing peaks. Partially purified tissue carnosinase and serum carnosinase preparations were mixed and chromatographed by the same procedure, resulting in a similar pattern of carnosine hydrolysis. The enzymes present in peaks I and II were shown to have apparent molecular weights of 90,000 and 160,000, respectively. Lenney et al. (1982) have previously shown that tissue carnosinase has an apparent molecular weight of 90,000 and did not hydrolyze homocarnosine, while serum carnosinase has an apparent molecular weight of 160,000 and hydrolyzed homocarnosine much more slowly than it hydrolyzed
carnosine. Also, the isoelectric points of brain and serum carnosinase were found to be approximately 5.6 and 4.7, respectively. Therefore, serum carnosinase would be expected to elute after brain carnosinase during DEAE-cellulose chromatography. It is concluded that peak I represents tissue carnosinase and peak II represents serum carnosinase. Furthermore, normal human brain apparently does not contain the enzyme homocarnosinase, since the serum carnosinase fractions were the only peak of activity against homocarnosine which could be detected in the DEAE-cellulose column eluate. Peak II (mol. wt. 160,000) differs greatly from hog kidney homocarnosinase, which has an apparent molecular weight of 57,000 (Lenney et al., 1977). Further evidence that homocarnosinase is not present in human brain is the inability to detect it after isoelectric focusing; hog kidney homocarnosinase has an isoelectric point of 5.6 and was found to be stable during focusing (Lenney et al., 1977). No serum carnosinase activity was detected after focusing because this enzyme is unstable at its isoelectric point (Lenney et al., 1982).

A brain extract, human serum, and a partially purified serum carnosinase all displayed similar optimum assay conditions. In CSF samples of different control patients, homocarnosine hydrolysis was significantly related to serum carnosinase activity, and a CSF sample from a homocarnosinosis patient was deficient in both of these
activities (Fig. 3). In addition, the enzyme hydrolyzing carnosine and homocarnosine in normal CSF samples was shown to have an apparent molecular weight identical with serum carnosinase. Thus, it is concluded that the homocarnosine-hydrolyzing activity of human brain and CSF is attributable to serum carnosinase.

In other human tissues the enzyme homocarnosinase was not detected by electrophoresis. While the larger of the two peaks (Rf = 0.41) in Fig. 4 undoubtedly represents tissue carnosinase, the smaller peak (Rf = 0.61) probably represents serum carnosinase. When serum was electrophoresed under the same conditions, the carnosinase Rf value averaged 0.58 (Lenney et al., 1982). The lack of detectable homocarnosine-hydrolyzing activity in gels following electrophoresis of various tissues is probably due to the small quantity of applied extract (25 μl). Twelve human tissues other than brain were low in homocarnosine-hydrolyzing activity and in rough agreement to the amount of trapped blood (Fig. 3); their activity may be attributable to trapped serum carnosinase.

Although homocarnosinase was not found in human tissues, it is present in hog kidney and in certain rat tissues (Lenney et al., 1977). Homocarnosinase must be a cellular enzyme in these animals because no hydrolysis of carnosine was detectable in rat or hog serum (Lenney et al., 1977, 1982).
Human brain contains approximately 15 times the amount of serum carnosinase expected on the basis of the content of trapped blood. Presumably the enzyme is within the neural tissues, rather than only within the blood vessels of the brain. Perhaps serum carnosinase crosses the blood-brain barrier by an unknown process and accumulates in the brain. Another possibility is that serum carnosinase is synthesized in the brain and secreted into the CSF, where it is eventually transported into the blood stream. Davson et al. (1973) showed that human serum albumin injected into a lateral ventricle of the rabbit soon appeared in the plasma.

When Perry and coworkers (Kish et al., 1979; Perry et al., 1979) assayed human brain samples for homocarnosine-hydrolyzing activity, their assay parameters were very similar to the optimum conditions for serum carnosinase (pH, substrate concentration, and cobalt as the activating metal ion). They reported data showing the rate of homocarnosine hydrolysis in cerebral cortex samples from four individuals averaged 0.306 μmoles/g/h (Kish et al., 1979). Their data is in good agreement with this study, where the homocarnosine-hydrolyzing activity of cerebral cortex averaged 0.296 μmoles/g/h. Therefore, Perry and coworkers (Kish et al., 1979; Perry et al., 1979) were probably measuring homocarnosine hydrolysis by serum carnosinase rather than by brain homocarnosinase.

It has been reported that an unidentified enzyme in normal human CSF converts the homocarnosine in CSF to GABA.
and histidine (Grove et al., 1982a&b). Since serum carnosinase is present in CSF, this appears to be the enzyme responsible for the hydrolysis. The activity of this enzyme in CSF was weak (Grove et al., 1982), perhaps because of low concentrations of the necessary activating metal ion.

Sera from homocarnosinosis patients, a CSF sample from one of these patients, and a brain biopsy sample from another of them were deficient in serum carnosinase, as measured by carnosine and homocarnosine hydrolysis. Thus, the elevated concentrations of homocarnosine reported to be present in CSF samples and a brain sample of homocarnosinosis patients (Gjessing and Sjaastad, 1974; Perry et al., 1979b) is attributable to a deficiency of serum carnosinase rather than brain homocarnosinase. Serum carnosinase deficiency is also responsible for the decreased capacity of these patients to metabolize carnosine and anserine after consuming a dinner rich in the two dipeptides (Lunde et al., 1982).

The mother of the homocarnosinosis patients also lacked serum carnosinase activity (Table I). It was reported that she also has a very high level of homocarnosine in her CSF (Sjaastad et al., 1976) and is apparently unable to metabolize anserine or carnosine (Lunde et al., 1982), although she was physically and mentally normal (Sjaastad et al., 1976). The father (age 77) of the homocarnosinosis patients has a rather low serum carnosinase activity, at the
lower end of the range observed for elderly out-patients (Table I). In most inherited metabolic disorders involving the complete or almost complete absence of an enzyme in affected homozygotes, one expects heterozygote carriers to have about 50% of the normal homozygote enzyme activity (Harris, 1980). These results suggest that the serum carnosinase deficiency is inherited as an autosomal recessive trait, with the mother being a homozygous carrier of the abnormal gene and the father being heterozygous.

Since the mother is physically normal, the lack of serum carnosinase and resultant elevation of homocarnosine in the CSF does not, by itself, appear to cause any neuropathological symptoms. Perry et al. (1979) pointed out that there is likely to be another hereditary disorder responsible for the neurological degeneration which was inherited as an autosomal recessive trait. Perhaps a deficiency in serum carnosinase might be necessary for the postulated abnormal gene to be expressed as a neurological disease.

Patients with homocarnosinosis display some features similar to those having carnosinemia, another metabolic disorder involving a deficiency of serum carnosinase (Perry et al., 1967; Perry and Hansen, 1968; Perry, 1974; van Heeswijk et al., 1969; Murphey et al., 1973; Fleisher et al., 1980; Wisniewski et al., 1981; Hartlage et al., 1982; Lunde, Sjaastad, and Gjessing, 1982). Neurological signs for both sets include progressive mental retardation and
spasticity. Like the mother of the homocarnosinosis patients, there are carnosinuric individuals lacking serum carnosinase but showing no neurological signs (Murphey et al., 1973; Gordon et al., 1977; Lunde et al., 1982). However, there are some distinct differences between them, e.g., retinal pigmentation appears only in the homocarnosinosis patients. There also appear to be several variant forms of carnosinemia (Burgess et al., 1975; Gordon et al., 1977; Fleisher et al., 1980; Hartlage et al., 1982). Furthermore, the onset of neurological signs in carnosinemia patients occurs within the first six months, and the children generally do not survive the first few years; the oldest patient reported was 13 years of age (Dolman, 1978). The earliest signs of spasticity in homocarnosinosis patients occur between 6 and 29 years of age (Sjaastad et al., 1976). It is conceivable that serum carnosinase deficiency increases an individual's susceptibility to a group of related neurological disorders; without the carnosinase deficiency the neurological dysfunction might not be clinically expressed.

Evidence has been presented in this chapter that all homocarnosine-hydrolyzing activity in normal human brain and CSF is attributable to serum carnosinase. Furthermore, a deficiency of this enzyme, rather than a brain homocarnosinase, is responsible for elevated homocarnosine levels in brain and CSF samples of homocarnosinosis patients.
CHAPTER III
CHARACTERIZATION OF HUMAN TISSUE CARNOsinASE

INTRODUCTION

Carnosinase was discovered by Hanson and Smith (1949), who found that the hog kidney enzyme was activated and stabilized by manganous ions. Wood (1957) showed that carnosinase was widely distributed in the tissues of the rat. Rosenberg (1960a,b,c) purified carnosinase from hog kidney and studied its activation and stabilization by metal ions. Lenney (1976) studied the specificity of purified hog kidney carnosinase and subsequently isolated a second carnosine-splitting enzyme (homocarnosinase) from hog kidney (Lenney et al., 1977). Wolos (1978) also reported the presence of two carnosine-hydrolyzing enzymes in the hog kidney. Margolis and coworkers (1979, 1983) isolated a manganese-independent low \( K_m \) carnosinase from mouse kidney, and studied the immunohistochemical localization of this enzyme in the tissues of the mouse. They also noted the presence of a manganese-dependent high \( K_m \) carnosinase in mouse tissues.

There have been very few studies on carnosinase of human tissues. Perry and coworkers (1968) first reported on the presence of serum carnosinase in normal human serum, noting its deficiency in children with carnosinemia. Human serum
carnosinase is reviewed in the previous chapter. Zoch and Muller (1971) demonstrated the presence of a manganese-activated carnosinase in extracts of human placenta. Murphey et al. (1972, 1973) measured tissue carnosinase activities of human kidney, liver and spleen, and reported that each tissue contained two electrophoretic forms of carnosinase. Lenney et al. (1982) characterized human serum carnosinase and showed it was different from human tissue carnosinase. The enzyme homocarnosinase was not detected in human tissues (Lenney et al., 1983; also Chapter II of this thesis). A review of the literature showed that no systematic study of human tissue carnosinase has been published.

In this chapter, human tissue carnosinase has been highly purified and characterized. This enzyme had an unusually high pH optimum and was strongly activated by dithiothreitol. It had a broader specificity than hog kidney carnosinase or human serum carnosinase. It also appears to be a true dipeptidase rather than an aminopeptidase because it hydrolyzes dipeptides but none of the closely related tripeptides.

MATERIALS AND METHODS

Materials
Peptides contained only the L-form for amino acids, except for 8-alanyl-D,L-leucine. Mono Q HR 5/5 column was
from Pharmacia Fine Chemicals (Piscataway, NJ) and homocarnosine and glycy1-L-histidyl-L-glycine were from Aldrich Chemical Co. (Milwaukee, WI). Carnosine, other di- and tripeptides, cytosolic and microsomal leucine aminopeptidases, and all other reagents were from Sigma Chemical Co. (St. Louis, MO). Bestatin, amastatin and arphaminine were gifts from Dr. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan); pancreatic dipeptidase was given by Dr. Y. Ito (Gifu College of Pharmacy, Gifu, Japan); and MK0791 was a gift from Dr. H. Kropp (Merck Institute, Rahway, NJ).

Enzyme assays

All assays using human tissue and plasma carnosinases were performed as described in Chapter II. Assays of hog kidney carnosinase were similar, except digest tubes contained 20 mM carnosine, 0.07 mM MnCl₂, 1 mM DTT, and 60 mM Tris-HCl buffer (pH 8.8). Assays of hog kidney homocarnosinase (Lenney et al., 1977) and human pancreatic dipeptidase (Ito et al., 1983) followed published methods, but the degree of hydrolysis of histidine-containing substrates was measured by the modified Ambrose method; hydrolysis of other substrates was measured by the ninhydrin method of Moore (1968).
Distribution in various tissues

Tissues (1.0 g) were homogenized in a mortar with a pestle using fine glass beads with 5 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM MnCl₂. The supernatant (10 min at 15,000 x g) was diluted 10-fold with this buffer and 0.10 ml was assayed for tissue carnosinase activity by the above procedure.

Electrophoresis

Polyacrylamide gel disc electrophoresis was carried out in tubes by the method of Davis (1964) using a stacking gel but no sample gel. After electrophoresis at 80°C for 2 h at 30 mA in the presence of 0.1 mM MnCl₂, the gel rod was cut into 5-mm sections. The gel sections were placed into borosilicate (12 x 75 mm) tubes and macerated using a glass rod. To each tube was added 0.10 ml of 250 mM sarcosine-HCl buffer (pH 9.5), 0.10 ml of 10 mM DTT, and 0.10 ml of 0.125 mM MnCl₂. The suspension was incubated with occasional shaking for 2 h at 30°C. Then 0.50 ml of 0.6 N trichloroacetic acid was added and the tubes were centrifuged. A portion (0.10 ml) of the supernatant was analyzed for histidine by the modified Ambrose method.

Tissue carnosinase was also subjected to electrophoresis in a 16 x 18 x 0.15 cm slab of polyacrylamide gel under the same conditions as above. After electrophoresis at 4°C for 2.5 h at 25 mA (450 V), a 2.5-cm
vertical strip was removed from the gel slab and stained overnight in 0.05% Coomassie blue dissolved in 45.4% methanol and 9.2% glacial acetic acid. The remainder of the slab was cut into 0.75-cm horizontal strips, and a 1-cm section of each strip was then assayed for carnosinase as described above. The vertical strip was destained in a solution containing 45.4% methanol and 9.2% glacial acetic acid; the $R_f$ values of the protein bands were determined.

The isoelectric point of tissue carnosinase was determined by isoelectric focusing in 7.5% polyacrylamide gel rods. The gels contained 2.4% ampholyte (pH range 3.5-10 or 5-7) and 0.1 mM MnCl$_2$. After focusing, the gels were cut into 5-mm sections and the enzyme was measured by the method described above.

**Purification of tissue carnosinase**

DEAE-cellulose (settled volume of 215 ml) was regenerated and equilibrated with 10 mM Tris-HCl (pH 7.0) containing 0.1 mM MnCl$_2$ and 0.02% NaN$_3$. Human kidney (40 g), obtained at autopsy, was cut into 1/2 inch cubes and homogenized in a blender with 210 ml of the buffer. The clarified supernatant (10 min at 8000 x g) was mixed with the DEAE-cellulose suspension with occasional stirring for 1 h at 22°C and the mixture was poured into a column (4 cm diameter). The bed was then washed with the same buffer until the absorbance at 280 nm dropped below 0.05. A 450-ml
linear gradient of NaCl (0-0.5 M) in the same buffer was passed through the column. The fractions containing carnosinase were pooled and concentrated to 32 ml by ultrafiltration on a Diaflo YM30 membrane.

Sephadex G-200 was equilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.1 mM MnCl₂ and 0.02% NaN₃ and was then poured into a 4-cm diameter column to a packed bed height of 75 cm. The carnosinase preparation (30 ml) was applied onto the column and eluted with the pH 7.4 buffer at 0.6 ml/min. The fractions containing carnosinase activity were pooled and concentrated as before to 12 ml. The concentrate was found by the method of Lowry et al. (1951) to contain 3.7 mg protein per ml.

A Mono-Q HR 5/5 column (Pharmacia), attached to a Beckman HPLC system, was used to further purify tissue carnosinase. The column was equilibrated with 20 mM Tris-HCl (pH 7.65) containing 10% glycerol and 0.1 mM MnCl₂. Volumes of 0.1 or 0.4 ml carnosinase preparation were chromatographed either isocratically (0.105 M NaCl in buffer) or with a 30-ml linear gradient (0-0.5 M NaCl in buffer) or section of the same gradient. Flow rate was 1.0 ml/min in all cases. To each eluted fraction was added 0.1 volume of 1 mM EDTA containing 2 mM MnCl₂. Fractions having carnosinase activity were employed in polyacrylamide gel electrophoresis and in substrate specificity assays.
RESULTS

**Effect of dithiothreitol**

Human tissue carnosinase was activated by dithiothreitol (DTT) and inactivated by p-hydroxymercuribenzoate, indicating that it is a cysteine peptidase. The amount of activation varied from 2- to 6-fold, presumably depending upon the degree of oxidation of the enzyme in the preparation employed. Maximum activity was obtained at a dithiothreitol concentration of 2 mM; this concentration was used in all routine assays.

**pH-activity curve**

In Fig. 5 the pH-activity curve of tissue carnosinase is compared with that of serum carnosinase. Kidney, brain and liver carnosinases all displayed very similar pH-activity curves. In the tissue carnosinase assay at pH 9.5, sarcosine or 2-amino-2-methyl-1,3-propanediol buffers gave maximal activity, NH$_4$OH-HCl gave 20% less activity, and borate was strongly inhibitory. A crude kidney extract and a partially purified brain preparation were analyzed under many different conditions at pH 7.2; the maximum activity attained was only 2% of that observed under standard assay conditions at pH 9.5.
Fig. 5. Effect of pH on the activity of tissue (O) and plasma (●) carnosinases. The enzyme preparations were DEAE-cellulose column eluates; 30-minute assays were done in the presence of 0.05 M Tris-0.05 M sarcosine-HCl buffer mixtures. pH values were measured during the assay.
Enzyme calibration curve

When various amounts of crude liver extract were employed in the assay for carnosinase, a nonlinear enzyme calibration curve was obtained (Fig. 6). The downward curvature observed with the nondialyzed extract suggests the presence of an endogenous reversible inhibitor(s) (Dixon and Webb 1979). After charcoal-facilitated dialysis of the extract, activity was increased and the calibration curve approached linearity, indicating almost complete removal of inhibitor. Partially purified kidney and brain carnosinase preparations produced strictly linear calibration curves.

In routine assays of tissues for carnosinase, we found that the use of dilute nondialyzed extracts usually gave higher activity per g of tissue than the use of more concentrated dialyzed extracts. In certain tissues, carnosinase activity declined during dialysis, probably because of proteolysis. The endogenous inhibitors will be described in Chapter IV.

Effect of metal ions on activity

Crude kidney and brain extracts were dialyzed at 4°C against various 1 mM metal salts at pH 7.0. The extracts dialyzed vs. MnCl₂ were very active, whereas those containing CoSO₄, CdCl₂, CaCl₂ or ZnSO₄ had little or no activity. In addition, MnCl₂ was much more effective than the other salts in reversing the inhibition of partially purified brain and kidney carnosinases by 0.2 mM EDTA.
Fig. 6. Effect of dialysis on the enzyme calibration curve of liver carnosinase. Ten grams of human liver were homogenized in 50 ml of 10 mM Tris-HCl (pH 7.5) containing 0.2 mM MnCl₂ and 0.02% NaN₃. Half of the supernatant (10 min at 5000 x g) was dialyzed overnight with stirring at 4°C against 900 ml of the above buffer containing 5 g of charcoal. The other half of the extract was stored at 4°C without dialysis. Various amounts of the dialyzed (●) and nondialyzed (O) extracts were analyzed for carnosinase activity.
Maximum activity of tissue carnosinase at 2 mM DTT and pH 9.5 was observed at approximately 0.05 mM MnCl₂; higher concentrations caused a marked decline in carnosinase activity. Thus, manganese was an activator or an inhibitor, depending upon the concentration.

Effect of metal ions and pH on stability

Partially purified brain carnosinase was heated in the presence of various metal ions (0.1 mM). As compared to a control with no metal added, MnCl₂ increased thermostability, FeSO₄, MgSO₄ and CaCl₂ had no effect, whereas CoCO₄, ZnSO₄ and CdCl₂ decreased stability. As shown in Fig. 7, maximum stability at 50°C was observed at pH 7.2-8.2. Thermostability varied slightly from one experiment to another, and no actual difference between kidney and brain carnosinases was apparent. Although Fig. 7 shows that stability at high pH values is poor, it was found that tissue carnosinase hydrolyzes carnosine at a constant rate in the standard assay, indicating that the enzyme is stable under assay conditions. It is well known that a substrate tends to increase the stability of its enzyme.

Effect of substrate concentration

Fig. 8 illustrates the effect of carnosine concentration on tissue carnosinase activity at 0.025 mM MnCl₂. The best fitting computer-generated hyperbola of the
Fig. 7. Effect of pH on the stability of tissue carnosinase at 50°C. Portions of DEAE-cellulose column eluates from human brain (O) and kidney (●) were heated for 30 min at 50°C in the presence of 0.8 mM MnCl₂ and a buffer mixture containing 5 mM acetate, 5 mM N-ethylmorpholine, and 5 mM sarcosine. Residual activity was measured using the standard assay procedure.
Fig. 8. Effect of substrate concentration on the activity of brain carnosinase. The data are plotted according to Michealis-Menton (inset) and also by a half-reciprocal method.
data in Fig. 8 gave a $K_m$ value of 10 mM. When the data were analyzed using a half-reciprocal plot, brain and kidney carnosinases were found to have a $K_m$ of approximately 10 mM. Recently, it was observed that doubling the concentration of MnCl$_2$ to 0.05 mM resulted in a $K_m$ shift to about 23 mM.

**Molecular weight**

When kidney or brain carnosinase was passed through a calibrated Sephadex G-200 column, each was found to have an apparent molecular weight of 90,000. This result agrees with the value obtained for human kidney carnosinase by Murphey et al. (1972).

**Electrophoresis**

Murphey et al. (1972) reported that human kidney, liver and spleen each contained two forms of carnosinase; they observed two peaks of activity when extracts of these tissues were subjected to starch block electrophoresis at pH 7.0. However, when we subjected extracts of kidney and liver to electrophoresis under similar conditions (Fig. 9), only one peak of activity was detected.

Crude extracts and partially purified preparations from kidney, brain and placenta were also subjected to non-denaturating polyacrylamide disc gel electrophoresis (gel pH 8.9). In each case, one large tissue carnosinase peak ($R_f = 0.38-0.45$) was observed. In addition, a very small peak
Fig. 9. Electrophoresis of kidney (○) and liver (●) carnosinases in a horizontal slab of Pevikon and Sephadex at pH 7.5. The slab was 24.5 x 11.0 x 0.5 cm made up of 80 g Pevikon C-870 and 4 g Sephadex G-75 in a 0.1 M Tris-HCl buffer containing 1 mM MnCl$_2$ at pH 7.5. Crude dialyzed extracts were run side by side with a plastic partition between the two lanes. Electrophoresis was on a cooled plate at 8°C for 14 h at 25 mA; the enzymes moved toward the anode. Enzyme recoveries were over 90%.
(R_f = 0.54-0.62) was detected, probably representing serum carnosinase present in trapped blood of the tissue sample (Lenney et al., 1983; Chapter II).

Extracts of kidney, brain and liver were also electrophoresed on vertical polyacrylamide and starch gel slabs (gel pH 8.8). In each case, a single carnosinase spot was seen when the Lewis and Harris (1967) dipeptidase detection procedure was employed.

**Isoelectric point**

When tissue carnosinase was subjected to isoelectric focusing in a polyacrylamide gel, a single peak of activity at pH 5.6 ± 0.1 was observed (Fig. 10). Partially purified kidney and brain preparations showed a single sharp peak whether narrow or broad range ampholytes (pH 3.5-10.0) were used. A similar result was obtained when the tissue enzyme was focused in a sucrose gradient. A minor peak representing serum carnosinase was not observed because this enzyme is unstable at its isoelectric point of 4.7 (Lenney et al., 1982). No peak of activity against homocarnosine was detectable.

**Inhibitors**

Table III shows the effects of ten inhibitors on the activity of tissue carnosinase. Bestatin, an inhibitor of leucine aminopeptidase and aminopeptidase B (Aoyagi et al.,
Fig. 10. Isoelectric focusing of kidney carnosinase in a polyacrylamide gel rod. A DEAE-cellulose column eluate was employed in a gel containing ampholytes having a pH range of 5-7. Focusing was for 3.5 h at 410 V at 8°C. Enzyme recoveries averaged 85%.
### TABLE III

**INHIBITION OF TISSUE CARNOSINASE BY VARIOUS COMPOUNDS**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.4 mM</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate *</td>
<td>0.4 mM</td>
<td>98</td>
</tr>
<tr>
<td>PhenylmethyIsulfonyl fluoride</td>
<td>1.0 mM</td>
<td>38</td>
</tr>
<tr>
<td>Bestatin</td>
<td>4.0 nM</td>
<td>50</td>
</tr>
<tr>
<td>Amastatin</td>
<td>13 μM</td>
<td>41</td>
</tr>
<tr>
<td>Leucine hydroxamate</td>
<td>80 μM</td>
<td>93</td>
</tr>
<tr>
<td>Alanyl-histidine</td>
<td>0.4 mM</td>
<td>98</td>
</tr>
<tr>
<td>Alanyl-leucine</td>
<td>0.4 mM</td>
<td>69</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>40 mM</td>
<td>49</td>
</tr>
<tr>
<td>CaSO$_4$</td>
<td>3 mM</td>
<td>35</td>
</tr>
</tbody>
</table>

* Dithiothreitol was omitted from experimental and control digests.
1977) was the most potent inhibitor, followed by amastatin. Bestatin contains leucine and has a backbone chain identical with that of carnosine; bestatin inhibition of carnosinase is the subject of Chapter V. A number of other dipeptides containing leucine were also inhibitory; these are discussed in detail in Chapter IV. Ala-his and gly-leu, substrates of carnosinase, inhibited the hydrolysis of carnosine. Phenylmethylsulfonyl fluoride, an inhibitor of serine proteinases, was inhibitory at 1 mM; this compound inhibits papain by reacting with its sulfhydryl groups (Whitaker and Perez-Villasenor, 1968). Homocarnosine and anserine, which were not hydrolyzed by carnosinase, did not inhibit carnosine hydrolysis.

Other compounds that were not inhibitory include:
arphaminine (10 μg/ml), leupeptin (5 μM), puromycin (4 μM), MK 0891, a dipeptidase inhibitor (10 μg/ml), bacitracin (140 μM), spermine (0.8 mM), spermidine (0.8 mM), putrescine (0.8 mM), cadaverine (0.8 mM), sodium citrate (4 mM), Na₂HPO₄ (10 mM), adenosine (0.2 mM), adenine (0.2 mM), guanosine (0.2 mM), and guanine (0.2 mM).

**Purification**

Human kidney carnosinase was purified by chromatography on columns of DEAE-cellulose, Sephadex G-200 and Mono-Q, as described in the Methods section. Two forms of tissue carnosinase were resolved on the Mono-Q column (Fig. 11).
Fig. 11. Mono-Q chromatography of a human kidney carnosinase preparation. The enzyme preparation (0.1 ml) was injected onto the column at the arrow. Periods of isocratic and gradient elution are designated.
Carnosine hydrolyzed (μmoles)

Time (min)

Absorption at 280 nm

Fraction No.
Human brain carnosinase exhibited a similar elution pattern. The enzyme was unstable after this step, but could be stabilized by adding EDTA in excess MnCl₂, suggesting that toxic metal ions had been picked up from the stainless steel tubing of the HPLC apparatus.

When human kidney carnosinase was isocratically chromatographed on Mono-Q, a more purified first peak of carnosinase was obtained. This was then concentrated by vacuum dialysis and was subjected to polyacrylamide gel electrophoresis at pH 8.9. Coomassie Blue staining revealed three protein bands ($R_f$ 0.23–0.28). In an unstained gel strip, carnosinase activity was detected in this region of the gel, but an exact correspondence of the enzyme to one of the protein bands was not established.

A major problem in isolating tissue carnosinase was the poor recovery of enzyme activity after gel electrophoresis. Several different techniques of extracting tissue carnosinase from the polyacrylamide gel were unsuccessful, including electrophoretic concentration over a dialysis membrane. Electrophoresis in ultra-low melting-point agarose was also unsuccessful because agarose inhibited the enzyme. When mashed sections of polyacrylamide gel were employed in the assay procedure, activity was detected. However, the highly purified tissue carnosinase was unstable in the gel, losing activity rapidly even after the strips had been adjusted to pH 7.2 and glycerol and MnCl₂ were added.
Specificity

Because of the difficulty involved in obtaining a stable preparation of pure carnosinase, an alternative method was employed to examine its specificity. In an isocratic Mono-Q experiment, fractions that preceded and included the first carnosinase peak were assayed against 13 different potential substrates. Because of the presence of contaminating enzyme(s), differences between substrates in baseline values were corrected by standardizing all changes in hydrolytic activities to the activity of the first fraction. Fig. 12 shows the resultant profiles of the relative rates of substrate hydrolysis. Some of the profiles were similar to the curve for carnosine; that is, the rate of substrate hydrolysis increased rapidly to a maximum at fraction #2 and then declined more gradually over the last two fractions. Such a profile indicates that a compound is hydrolyzed by carnosinase, since it is unlikely that another enzyme would also elute in exactly the same pattern. From Fig. 12, it appears that kidney carnosinase was able to hydrolyze his-ala, gly-his, and gly-leu faster than carnosine, and to hydrolyze his-gly and ala-his less rapidly than carnosine. No hydrolytic activity was detected with any of the tripeptides tested, nor with anserine, N-acetyl-his or benzylxycarbonyl-glu-tyr. Tissue carnosinase also does not split homocarnosine (Lenney et al., 1983; Chapter II).
Fig. 12. Substrate specificity of tissue carnosinase. Fractions #1 - #4 from an isocratic Mono-Q chromatograph were assayed in the presence of different peptides, each at 1.2 mM, using digests and blanks. Two methods of measuring hydrolysis products were employed: the increase in fluorescence is roughly proportional to the amount of histidine released, whereas the ninhydrin color development is related to free amino acids released. Results are plotted as the change in fluorescence or ninhydrin color relative to the value at fraction #1. Peptides tested were: gly-L-his (GH), carnosine (C), L-his-gly (HG), L-ala-L-his (AH), gly-L-his-L-lys (GHL), gly-L-his-gly (GHG), L-his-L-ala (HA), gly-gly (GG), gly-L-leu (GL), carbobenzoxy-L-glu-L-tyr (CGT), N-acetyl-L-his (NAH), anserine (A), and gly-gly-gly (GGG).
A sample of highly purified human pancreatic dipeptidase (Ito et al., 1983) was active in hydrolyzing gly-his, ala-his and gly-leu but was unable to hydrolyze carnosine. This enzyme was inhibited by MK 0791 (10 μg/ml) but was unaffected by bestatin (1 μg/ml). Cytosolic and microsomal porcine kidney leucine aminopeptidases also failed to hydrolyze carnosine.

**Distribution of tissue carnosinase**

Dilute extracts of 17 human tissues were analyzed for carnosinase (Table IV) and activity was found in each. Kidney, liver, spleen and brain were the richest sources of this enzyme. Very little was found in the uterus, although this organ is an excellent source of carnosinase in the rat (Lenney, 1976). The enzyme in washed erythrocytes was tissue carnosinase rather than serum carnosinase, judging from its relative activities in the two assay methods. The concentration of carnosinase in the kidney and liver were about 10 times higher than the values reported by Murphey et al. (1973).

**Subcellular localization**

Fresh rat kidney and liver and mouse liver were homogenized in 0.25 M sucrose and fractionated centrifugally (Liao and Lenney, 1984). The manganese-dependent carnosinase was found in the 100,000g supernatant and not in the
## TABLE IV

### DISTRIBUTION OF TISSUE CARNO SINASE IN HUMAN TISSUES

<table>
<thead>
<tr>
<th>Tissue</th>
<th>This study</th>
<th>Results of others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carnosinase activity</td>
<td>µmol/g per h ± S.D. (n)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75.1 ± 6.0 (3) a</td>
</tr>
<tr>
<td>Kidney</td>
<td>790 ± 220 (6)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>300 ± 60 (4)</td>
<td>23.0 (2) a</td>
</tr>
<tr>
<td>Spleen</td>
<td>220 ± 110 (6)</td>
<td>70.4 (2) a</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>105 ± 57 (4)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>73 ± 31 (4)</td>
<td></td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>61 (1)</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>49 ± 23 (3)</td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>49 (1)</td>
<td></td>
</tr>
<tr>
<td>Ovaries *</td>
<td>45 (1)</td>
<td></td>
</tr>
<tr>
<td>Testes</td>
<td>40 ± 19 (3)</td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>27 (2)</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>25 (2)</td>
<td>n.d. (1) a</td>
</tr>
<tr>
<td>Heart</td>
<td>21 (2)</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>15 (2)</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes **</td>
<td>12 (1)</td>
<td></td>
</tr>
<tr>
<td>Gall bladder</td>
<td>11 (1)</td>
<td></td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>10 (1)</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>9 (1)</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>6 (1)</td>
<td></td>
</tr>
</tbody>
</table>

a Murphey et al. (1973), assuming 128 mg total protein per g of fresh tissue (Kirkham and Allfrey, 1972).

b Fleisher et al. (1980).

n.d. = none detected.

* Ovaries were extracted by use of a blender and analyzed after charcoal-facilitated dialysis. All other tissues in this study were treated as described in "Methods".

** Erythrocytes were washed three times with 0.9% NaCl.
particulate fraction. Cathepsin B served as a marker for the particulate fraction. Very fresh human tissues were not available, so frozen human placenta and kidney were fractionated by a similar procedure. Again, the carnosinase was found only in the cytosolic fraction.

DISCUSSION

In Table V the properties of human tissue and serum carnosinases are compared. Because of substantial differences between these enzymes, they should be regarded as isozymes (different gene products) rather than multiple forms of the same enzyme. Tissue carnosinase has a broader specificity than serum carnosinase or hog kidney carnosinase (Lenney, 1976).

Tissue carnosinase was strongly inhibited by bestatin, which is regarded as an inhibitor of certain aminopeptidases (Aoyagi et al., 1977). Nonetheless, tissue carnosinase appears to be a dipeptidase, rather than an aminopeptidase. Although it was very active in hydrolyzing gly-his, it failed to hydrolyze either of the peptide bonds in the closely related tripeptides gly-his-gly, gly-his-lys or gly-gly-gly. Another dipeptidase inhibited by bestatin was an enzyme from Streptococcus cremoris (Hwang et al., 1982).

In the literature it has tacitly been assumed that human serum and tissue carnosinases are similar to hog
TABLE V

COMPARISON OF HUMAN CARNOSINASES

<table>
<thead>
<tr>
<th>Property</th>
<th>Tissue carnosinase</th>
<th>Serum carnosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>broad</td>
<td>narrow</td>
</tr>
<tr>
<td>Cysteine enzyme?</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>9.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>5.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>90,000</td>
<td>160,000</td>
</tr>
<tr>
<td>Km – carnosine</td>
<td>10 mM</td>
<td>4 mM</td>
</tr>
<tr>
<td>Km – homocarnosine</td>
<td>---</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Activated by:</td>
<td>Mn</td>
<td>Cd</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td></td>
<td>citrate</td>
</tr>
<tr>
<td>Inhibited by:</td>
<td>EDTA</td>
<td>EDTA</td>
</tr>
<tr>
<td>pHMB</td>
<td>dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>bestatin</td>
<td>bestatin (weak)</td>
<td></td>
</tr>
<tr>
<td>borate</td>
<td>Tris</td>
<td></td>
</tr>
</tbody>
</table>
kidney carnosinase. The human enzymes have been analyzed under the conditions recommended for the porcine enzyme by Hanson and Smith (1949), i.e., pH 8.0, Tris buffer, 37°C, Mn$^{2+}$ as the activating metal ion. By reducing tissue carnosinase with dithiothreitol and analyzing at pH 9.5, the activity of this enzyme was increased 5-15 fold. Furthermore, crude extracts should be assayed using dilute solutions, otherwise endogenous inhibitors will depress the activity (Fig. 6).

Tissue carnosinase has a small amount of activity at pH 7.2; this increases sharply as the pH is raised to 9.5. Therefore it is postulated that localized pH changes within the cytosol could serve to regulate the activity of this enzyme. It is also likely that the process of optimizing the enzyme activity has resulted in an alkaline shift of the pH-activity curve; i.e., the pH optimum in vivo may be closer to the physiological pH than under the optimized conditions. Such alkaline shifts caused by anions have been described for other enzymes (Dixon and Webb, 1979, p.396). Also, Hanson and Smith (1949) and Rosenberg (1960b) showed a major shift of the pH-activity curve to the right when hog kidney carnosinase was activated by Mn$^{2+}$ rather than by Zn$^{2+}$. Therefore, the pH-activity curve in Fig. 5 is not strictly valid for in vivo carnosinase.

Manganese inhibits tissue carnosinase at concentrations in excess of its optimum (0.05 mM). Neither hog kidney
carnosinase (Rosenberg, 1960b) nor mouse kidney manganese-dependent carnosinase (Margolis and Grillo, 1984) are inhibited by manganese at concentrations up to 1 mM; both the hog and the mouse enzyme attained 75% of maximum activity at approximately 0.2 mM Mn$^{2+}$.

The enzyme homocarnosinase was not detected in human tissues; serum carnosinase appears to be the only human enzyme capable of splitting homocarnosine (Lenney et al., 1983; Chapter II). Also undetected in human tissues was the manganese-independent low $K_m$ carnosinase reported by Margolis and Grillo (1984) to be in certain strains of mice. Hog and rat tissues contain homocarnosinase and the manganese-dependent and independent carnosinases, but these animals do not have serum carnosinase. Margolis et al. (1983) mentioned that the manganese-dependent carnosinase of mouse tissues was partially membrane-associated, although no data were presented. Ganapathy and Leibach (1982) postulated the existence of a membrane-bound carnosinase in rabbit kidney. However, we were unable to detect particulate carnosinase activity in mouse, rat or human tissues. In contrast to the mouse (Margolis et al., 1979) the data presented in this study indicate that human kidney, brain and liver carnosinases are identical.

Murphey et al. (1972) reported that human kidney, liver and spleen each contained two forms of carnosinase which
were separable by starch block electrophoresis. One of these forms had an isoelectric point of approximately 7.0. We employed a similar method plus five other electrophoretic techniques and observed only one form of tissue carnosinase (pI 5.6). The reason for this discrepancy is not known. However, two forms of this enzyme were separated by means of high-resolution anion exchange chromatography (Fig. 11). The second form had a pI below 6.0 and there was preliminary evidence that it was derived from the first form by proteolysis; both forms were similar in molecular weight, were labile during purification through Mono-Q, were inhibited by extremely low concentrations of bestatin (Chapter V), and were 50% inhibited by 0.2 mM leucine (Chapter IV).

Human tissue carnosinase has been characterized in this chapter and distinguished from other carnosine-hydrolyzing mammalian enzymes. By using optimum conditions for its activity, carnosinase activity in tissue extracts were found to be about 10 times that previously reported. Although an active preparation of the pure enzyme was not obtained, a method of specificity determination which minimized interference of other enzymes indicated it to be a true dipeptidase.
CHAPTER IV
ENDOGENOUS INHIBITORS OF TISSUE CARNOSINASE

INTRODUCTION

In recent years it has become apparent that endogenous inhibitors of proteinases play a crucial role in the regulation or limitation of protein degradation within tissues and cells. This subject has been reviewed in depth by Lenney (1980) and Laskowski and Kato (1980). Excessive tissue destruction may sometimes result from a deficiency of endogenous endopeptidase inhibitors. For example, an inherited deficiency of alpha-1-antitrypsin is linked to early-onset emphysema (Laurell and Eriksson, 1962) and to a specific type of liver cirrhosis in children (Sharp, 1976) and adults (Morse, 1978). Drugs which inhibit proteinases are potentially valuable in the treatment of emphysema, arthritis, glomerulonephritis, periodontitis, atherosclerosis and muscular dystrophy (Baugh and Schnebli, 1980). Some inhibitors may, in addition to their protective function, play a regulatory role in the control of proteinase activity (Lenney, 1980). In spite of the increasing interest in proteinase inhibitors, very little has been reported concerning endogenous inhibitors of exopeptidases (Laskowski and Kato, 1980).

Chapter III describes the discovery of thermostable dialyzable inhibitor(s) of tissue carnosinase in human
tissues. In this chapter the tissue distribution of carnosinase inhibitors has been examined, an inhibitor has been isolated and identified as L-leucine, and some characteristics of the inhibition have been explored. To my knowledge, this is the first report of the isolation of an endogenous dipeptidase inhibitor.

**MATERIALS AND METHODS**

**Materials**
Silica gel thin-layer chromatography plates (Si250F) were from J.T. Baker (Philipsburg, NJ). Silica gel powder for column chromatography (S-9258) was from Sigma Chemical Company (St. Louis, MO). All other reagents were of reagent grade or were from sources listed in previous chapters.

**Carnosinase preparation**
Unless specified otherwise, the source of tissue carnosinase for assays was a partially purified preparation. Human cerebral cortex (136 g) was homogenized for 1 min in a blender with 300 ml of 10 mM Tris-HCl, pH 8.1, containing 0.05 M NaCl and 0.1 mM MnCl₂. The clarified supernatant (20 min at 15,000 x g) was adjusted to pH 8.1 with dropwise addition of 1 M NaOH and was applied to a 2.1 x 34.8 cm bed of DEAE-cellulose which had been equilibrated with the Tris
buffer. The column was washed with this buffer until the absorbance of the eluate at 280 nm dropped below 0.05. A 450-ml linear NaCl gradient (0.05-0.45 M) in the same buffer was passed through the bed at 0.45 ml/min. The procedure was conducted at 22°C. The fractions containing the tissue carnosinase (95 ml) were pooled and concentrated to 10 ml by ultrafiltration on a Diaflo YM30 membrane. Ten ml of glycerol was added and the mixture was stored at -18°C, at which temperature the enzyme was stable for many months.

**Enzyme and inhibitor assays**

The procedure for measuring the activity of tissue carnosinase was described in Chapter II. To measure inhibitory activity, 0.10 ml of an inhibitor preparation (or 0.10 ml of H2O for control) plus 0.10 ml of 250 mM sarcosine-HCl buffer (pH 9.5) was substituted for 0.20 ml of 125 mM sarcosine-HCl buffer (pH 9.5). To determine the percent inhibition, the amount of histidine released from carnosine in the presence of inhibitor was compared to that in the absence of inhibitor, with all other assay conditions equal.

**Measurements of carnosinase inhibitor in tissues**

Normal human tissues were obtained at autopsy (5-20 h after death) and stored at -70°C. Samples (10.0 g) of various tissues were homogenized for 1 min at 22°C in a
Virtis homogenizer with 50.0 ml of 10 mM Tris-HCl buffer, pH 7.5. Homogenates were heated at 90°C for 10 min, and centrifuged to clarity. The extract (0.1 ml) was then combined with the quantity of tissue carnosinase that, without inhibitor, is able to split 1.0 μmole of carnosine per hour. This digest and a control without inhibitor were assayed for residual enzyme activity.

**Thin-layer chromatography**

Samples of a carnosinase inhibitor preparation or standards were applied onto a 5 x 20 cm plate coated with a 0.25 mm layer of silica gel (hardened surface) by touching filled 3-μl capillary pipettes to the surface 2 cm from the base and 1 cm apart from each other. Each spot was dried completely by using warm air flow between repeated applications. The plate was developed with freshly prepared n-butanol/acetic acid/water (4:1:1) in a small vapor-saturated chamber at 22°C until the solvent front had migrated about 15 cm. Ninhydrin (0.2%) in acetone was sprayed onto the appropriate lanes of the air-dried plate, while the other lanes were shielded from the spray. The TLC plate was then heated at 60°C for 30 min, the ninhydrin-positive spots were outlined, and their R_{sp} values were measured.

To assay a lane of a TLC plate for the location of carnosinase inhibitors, silica gel in areas corresponding to
ninhydrin-positive spots and to adjacent and blank areas were scraped into borosilicate tubes and ground thoroughly with a rounded glass rod in 0.4 ml of distilled water. A portion (0.2 ml) of the clarified supernatant (20 min at 2000 x g) was assayed for inhibitor. Because the CaSO₄ binder was slightly inhibitory, control regions without inhibitor were assayed as blanks and the estimated inhibition due to binder was subtracted from the total inhibition for each area.

**Purification of carnosinase inhibitor**

A sample of normal human liver (200 g) was cut into 1-cm cubes, separated from coarse connective tissue, and homogenized for 2 min with 200 ml of H₂O in a blender. The homogenate was heated at 90°C for 30 min and the clarified supernatant (30 min at 16,000 x g) was adjusted with 1 N NaOH to pH 8.6 and applied onto a 4 x 31 cm bed of DEAE-cellulose equilibrated with a buffer of 5 mM NH₄OH brought to pH 8.9 with acetic acid. 500 ml of buffer was then passed through the column, followed by a NaCl gradient (0-0.5 M) in the buffer. The unadsorbed fraction, which contained all the inhibitor, was adjusted to pH 5.0 with acetic acid and applied onto a 4 x 48 cm bed of CM-cellulose equilibrated with a buffer of 10 mM acetic acid brought to pH 4.5 with NH₄OH. After about 800 ml of buffer eluted, a NaCl gradient (0-0.5 M) was passed through the column. The
carnosinase inhibitor, present in 340 ml of unadsorbed eluate, was lyophilized to dryness, redissolved in 12.5 ml in H₂O, and chromatographed on Sephadex G-25 (1.5 x 50 cm) equilibrated with distilled H₂O. The inhibitor, eluting in a volume of 44 ml (Ve/Vo approximately 2.0), was then adjusted to pH 8.5 with NH₄OH and NaOH.

A 1 x 103 cm column of the strong anion exchanger, AG1-X-8 in the chloride form, was treated with 5 mM NH₄OH until the eluate pH exceeded 8.0. The inhibitor preparation was applied to the bed. The column was then rinsed with 120 ml of 5 mM NH₄OH at 0.5 ml/min, at which volume the absorption of the eluate at 280 nm had dropped to below 1.0. Following this, 800 ml of 10 mM HCl was passed through the bed. The fractions were assayed for carnosinase inhibition and the most active peak was lyophilized.

Dry silica gel (15-40 μm, mean pore diam 6 nm) was suspended in butanol/acetic acid/H₂O (4:1:1) with three stirrings, allowed to equilibrate overnight, and poured into a 1.6 x 100 cm glass column to a bed height of 91 cm. The column was then rinsed for 48 h with the solvent at 0.07 ml/min. The lyophilized inhibitor preparation was dissolved in 60% ethanol and mixed with 2 ml of dry silica beads. This suspension was then dried at 75°C, layered onto the top of the packed column, and carefully saturated with the butanol/acetic acid/H₂O solvent. (When this three-component solvent was used to dissolve the lyophilized inhibitor, it
had developed two phases, both of which contained substantial inhibitory activity.) The butanol/acetic acid/H₂O solvent was then passed through the column and the eluate was collected in 10-minute 0.7-ml fractions. A 25-μl portion of each fraction was assayed for ninhydrin reactivity by the method of Moore (1968). An additional 50 μl of each was assayed for inhibitor. The most active peak of inhibition was then lyophilized, dissolved in H₂O, and relyophilized.

Approximately half of the dried inhibitor preparation (12.6 mg) was dissolved in 0.6 ml of H₂O and subjected to isocratic HPLC on a 1 x 25 cm column of Ultrasphere 5 μm ODS using 0.1 M triethylamine in 0.122 M acetic acid (pH 5.3) as the eluant (flow rate 4 ml/min). Peaks absorbing at 222 nm were collected and assayed for inhibitory activity. The fraction containing the most activity was rechromatographed under identical conditions to obtain 1.4 mg of a pure carnosinase inhibitor.

**Nuclear magnetic resonance**

Proton NMR spectra were obtained at 300 MHz in D₂O with a Nicolet NT-300 spectrometer. Chemical shifts are reported in δ units (ppm) relative to DHO (4.65 pmm) as internal standard.

**Amino acid analysis**

A heated and clarified liver extract was prepared as before and was then lyophilized and taken up in 4%
sulfosalicylic acid (3.3 g original wet liver per ml). The suspension was centrifuged (5,000 x g for 20 min) and the supernatant was diluted 5-fold in the starting buffer of the amino acid analyzer. The sample (20 μl) was injected onto a Dionex D-300 amino acid analyzer, an HPLC column containing 5-μm diameter sulfonated polystyrene beads. The chromatograph was performed according to the manufacturer's instructions, employing sodium citrate buffers and a ninhydrin reagent. Peak areas of ninhydrin color development were converted into umoles of amino acids and the concentrations in the original extract were calculated.

RESULTS

**pH-stability**

A crude preparation of carnosinase inhibitor was heated at 100°C for 10 min at various pH values and then assayed for inhibitory activity. The inhibitor was completely thermostable over a pH range of 1 to 11.

**Inhibitor content of human tissues**

Crude extracts of 14 different human tissues were heated to inactivate the carnosinase and were then assayed for inhibitor using a fixed amount of carnosinase preparation (Table VI). The quantity of inhibitor varied considerably
<table>
<thead>
<tr>
<th>Tissue</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>96 (2)</td>
</tr>
<tr>
<td>Spleen</td>
<td>88 (2)</td>
</tr>
<tr>
<td>Stomach</td>
<td>85 (2)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>69 (2)</td>
</tr>
<tr>
<td>Lung</td>
<td>69 (2)</td>
</tr>
<tr>
<td>Liver</td>
<td>64 (1)</td>
</tr>
<tr>
<td>Kidney</td>
<td>41 (1)</td>
</tr>
<tr>
<td>Pectoralis muscle</td>
<td>41 (1)</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>29 (1)</td>
</tr>
<tr>
<td>Ovaries</td>
<td>27 (1)</td>
</tr>
<tr>
<td>Heart</td>
<td>24 (1)</td>
</tr>
<tr>
<td>Uterus</td>
<td>22 (1)</td>
</tr>
<tr>
<td>Testes</td>
<td>21 (1)</td>
</tr>
<tr>
<td>Brain</td>
<td>17 (2)</td>
</tr>
</tbody>
</table>

Number of extractions and assays is shown in parentheses. The extract assayed represents 17 mg of tissue.
from one tissue to another; pancreas, spleen and stomach contained the highest amounts, followed by small intestine, lung and liver. Brain contained the least amount of activity, but no tissue analyzed was completely lacking inhibitor.

**Purification**

Because liver contained a substantial amount of inhibitor and was available in ample quantity, it was chosen as the source for purification. The first step, heating the crude extract, served to remove large amounts of protein by denaturation and also destroyed the carnosinase so that the inhibitory activity could be measured. Effective separation from many charged constituents was achieved by passage of the inhibitor fraction through DEAE-cellulose and CM-cellulose. Gel filtration through Sephadex G-25 gave further purification and indicated a molecular weight of less than 1000. When this eluate was then chromatographed on a strong anion exchange resin, several peaks of inhibitory activity eluted (Fig. 13). The first peak, which contained the most activity, was chosen for additional purification.

A small amount of this inhibitor peak was subjected to silica gel TLC with butanol/acetic acid/H₂O (4:1:1). Fig. 14 illustrates the development pattern of the plate when examined for ninhydrin reactivity and carnosinase
Fig. 13. Chromatography of carnosinase inhibitors on the strong anion exchange resin, AGl-X8. The eluted fractions were measured for inhibitory activity per 0.1 ml (———), absorption at 280 nm (---) and pH (-----).
Fig. 14. Thin-layer chromatography of carnosinase inhibitor. Values of carnosinase inhibition represent the averages from two lanes.
inhibition. The ninhydrin-positive spot having the highest 
$R_f$, well separated from the other spots, corresponded to the 
TLC area of greatest inhibition, suggesting that the 
inhibitor is ninhydrin-positive and that it may be greatly 
purified by silica gel column chromatography. Fig. 15 shows 
the elution profile of the inhibitor on such a column. As 
in TLC, the ninhydrin-positive peak with the greatest 
mobility corresponded to the largest inhibitory activity. 
When samples of the first and second $A_{570}$ peaks from this 
elution were developed on TLC, the $R_f$ values matched the 
first and second spots in Figure 14.

Reversed-phase HPLC of the inhibitor peak from the 
silica gel column revealed at least 10 separate peaks 
absorbing at 222 nm (Fig. 16A). The most active fraction 
was rechromatographed under the same conditions (Fig. 16B); 
the pure inhibitor obtained was ninhydrin-positive.

**Identification of L-leucine**

The inhibitor purified on HPLC was identified as 
L-leucine. It was noticed that leucine had the same $R_f$ as 
the inhibitor when subjected to TLC and that L-leucine also 
showed inhibitory activity. When leucine was injected onto 
the HPLC column, it had the same retention time as the 
carnosinase inhibitor peak shown in Fig. 16B. The 
concentration of inhibitor that results in a 50% inhibition 
under the standard assay conditions (IC$_{50}$) was measured to
Fig. 15. Silica gel column chromatography of carnosinase inhibitor. The eluted fractions were assayed for carnosinase inhibitory activity per 0.05 ml (O) and for ninhydrin-positive compounds (●).
Fig. 16. Reversed-phase HPLC of the major carnosinase inhibitor peak after passage through silica gel.

A. Representative chromatograph of the lyophilized carnosinase inhibitor preparation (1.6 mg). The sample in distilled H₂O was isocratically chromatographed as described in "Methods". Peaks absorbing at 222 nm were collected in fractions. Portions (0.025 ml) were twice lyophilized, and assayed for inhibitory activity against tissue carnosinase. The arrow indicates the time of injection.

B. Rechromatograph of the carnosinase inhibitor. An additional 11 mg of inhibitor preparation from the silica gel column eluate was chromatographed in four runs as above; the peaks corresponding to fraction #5 (Fig. 16A) were pooled, twice lyophilized and dissolved in the triethylamine-acetic acid buffer. This was then rechromatographed in three replicate runs on HPLC under the same conditions as before. Shown here is a representative chromatograph and the inhibitory activity of 0.01 ml of the fraction. The arrow indicates the time of injection.
be 25 μg/ml for L-leucine and 26 μg/ml for the HPLC-purified inhibitor. In addition, the proton NMR spectra of the inhibitor, having multiplet peaks at δ 3.565, 1.542, and 0.780 ppm, agreed with the pattern for leucine.

**Inhibition by amino acids and related compounds**

All of the common amino acids and several related compounds were assayed for carnosinase inhibition (Table VII). L-leucine was the most active of the common amino acids and had an IC₅₀ equal to 0.2 mM. In addition, L-leucine hydroxymate at 0.08 mM was quite potent against tissue carnosinase (93% inhibition); this is on a comparable scale to its inhibition of leucine aminopeptidase (Kᵢ = 14 μm) (Chan et al., 1982).

The products of carnosine hydrolysis (histidine and β-alanine) did not inhibit tissue carnosinase, nor did the charged amino acids. When the same amino acid solutions of Table VI were tested against serum carnosinase, only L-cysteine was found to show more than 15% inhibition (88%).

**Amino acid content of a liver extract**

The quantity of inhibitory amino acids was determined for a human liver extract using an HPLC amino acid analyzer. The concentration of leu, val, and isl were about 1.8 times the amount that was expected based on the literature values of free amino acid content of monkey livers (Enwonwu and
### TABLE VII

**INHIBITION OF TISSUE CARNOSINASE BY AMINO ACIDS AND RELATED COMPOUNDS**

<table>
<thead>
<tr>
<th>Compound (1 mM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-leucine</td>
<td>87</td>
</tr>
<tr>
<td>L-cystine</td>
<td>66</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>63</td>
</tr>
<tr>
<td>DL-norvaline</td>
<td>56</td>
</tr>
<tr>
<td>L-valine</td>
<td>55</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>52</td>
</tr>
<tr>
<td>L-methionine</td>
<td>52</td>
</tr>
<tr>
<td>DL-norleucine</td>
<td>43</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>41</td>
</tr>
<tr>
<td>DL-isoleucine</td>
<td>26</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>19</td>
</tr>
<tr>
<td>L-alanine</td>
<td>18</td>
</tr>
<tr>
<td>glycine</td>
<td>15</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>12</td>
</tr>
<tr>
<td>L-arginine</td>
<td>12</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>11</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>11</td>
</tr>
</tbody>
</table>

Values reported are the averages of duplicate assays at 1 mM digest concentration. Compounds that were found to inhibit carnosinase by less than 10% are, in order of decreasing inhibition: taurine, L-glutamic acid, L-proline, L-serine, N-acetyl-L-aspartic acid, L-lysine, D-Leucine, N-acetyl-L-leucine, GABA, β-alanine, DL-threonine, L-glutamine, L-aspartic acid, histamine, L-histidine, and 1-methyl-L-histidine.
Worthington, 1974); cystine concentration was 3.7 times the expected value from the literature. Yet, when the measured amino acid concentrations were divided by their estimated inhibitory potencies (μmoles/IC₅₀), the sum total of inhibitory activity in the extract was only one fourth of the measured inhibitory potency of the extract. In order to check whether this discrepancy may be attributed to synergistic inhibition by combined amino acids, a solution containing amino acids at the expected composition from the literature was assayed. No synergism was detected. Therefore, leucine and other amino acids contributed about one fourth of the inhibitory activity of the liver extract, and at least one other inhibitor must have been present. Since about three fourths of the original extract activity was present in the 4% sulfosalicylic acid supernatant, most of the remaining inhibition probably resulted from one or more low molecular weight compounds, such as dipeptides.

**Inhibition by dipeptides and related compounds**

Table VIII shows the inhibition of tissue carnosinase by 0.4 mM solutions of eleven dipeptides. L-ala-L-his was most active in blocking carnosine hydrolysis. Since this dipeptide is also slowly hydrolyzed by tissue carnosinase (Chapter III), it is a competitive substrate. The dipeptides containing L-leucine were moderately active inhibitors. The degrees of inhibition by L-gly-L-leu (also a substrate) and
TABLE VIII
INHIBITION OF TISSUE CARNOSINASE BY VARIOUS DIPEPTIDES

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanyl-L-histidine</td>
<td>98 (1)</td>
</tr>
<tr>
<td>L-leucyl-L-leucine</td>
<td>85 (2)</td>
</tr>
<tr>
<td>L-alanyll-L-leucine</td>
<td>77 (2)</td>
</tr>
<tr>
<td>L-glycyl-L-leucine</td>
<td>73 (1)</td>
</tr>
<tr>
<td>L-alanyl-L-tryptophan</td>
<td>56 (1)</td>
</tr>
<tr>
<td>D-alanyl-DL-leucine</td>
<td>34 (2)</td>
</tr>
<tr>
<td>D-alanyl-L-tryptophan</td>
<td>23 (1)</td>
</tr>
<tr>
<td>D-alanyl-L-alanine</td>
<td>13 (1)</td>
</tr>
<tr>
<td>anserine</td>
<td>5 (1)</td>
</tr>
<tr>
<td>D-alanyl-L-glycine</td>
<td>3 (1)</td>
</tr>
<tr>
<td>homocarnosine</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

Number of determinations is in parentheses.
by L-ala-L-leu were comparable to the inhibition by the same concentration of L-leucine (74%), whereas inhibition by L-gly-L-leu was greater.

Homocarnosine and anserine did not show inhibition and were not substrates of tissue carnosinase. On the other hand, both of these imidazole dipeptides are substrates of serum carnosinase; 0.8 mM homocarnosine inhibited this isozyme by 20%, and 1 mM anserine inhibited it by 89%.

Bestatin, a dipeptide which inhibits certain aminopeptidases (Aoyagi et al., 1977), was an extremely potent inhibitor of tissue carnosinase ($IC_{50} = 4 \text{nM}$). Bestatin inhibition of carnosinase will be the subject of Chapter V.

**Effects of Mn$^{2+}$ on inhibition by amino acids**

L-leucine (0.2 mM) and L-cysteine (1 mM) were analyzed for their ability to inhibit carnosinase at different MnCl$_2$ concentrations. Tissue carnosinase was most active at 0.05 mM MnCl$_2$, with or without L-leucine (Fig. 17A); however, in the presence of L-cysteine, the optimum MnCl$_2$ concentration was elevated to 0.2 mM. The percent inhibition was calculated for each data point and plotted against MnCl$_2$ concentration in Fig. 17B. Inhibition by L-cysteine decreased as MnCl$_2$ concentrations increased above 0.02 mM. For L-leucine, the inhibition was substantially reduced by MnCl$_2$ concentrations lower than 0.02 mM but was not much affected by greater concentrations.
Fig. 17. Influence of MnCl₂ on the inhibitory activities of L-leucine and L-cysteine.

A. Effect of MnCl₂ concentration on carnosinase activity with and without L-leucine or L-cysteine. Carnosinase activity was determined over a wide range of MnCl₂ concentration in the presence of 0.2 mM L-leucine (O) or 1 mM L-cysteine (Θ) or in their absence as control (●). Values shown are averages of duplicate determinations. The arrow indicates the MnCl₂ concentration in the standard assay procedure.

B. Percent inhibition of carnosinase by L-leucine or L-cysteine as a function of MnCl₂ concentration. The data shown in Fig. 17A were analyzed to determine the percent inhibition of carnosinase by 0.2 mM L-leucine (O) and by 1 mM L-cysteine (Θ). Percent inhibition is the decrease between control and inhibitor activities divided by the control activity, all at the same MnCl₂ concentration. The arrow indicates the MnCl₂ concentration in the standard assay procedure.
Human serum carnosinase was strongly inhibited by 1 mM L-cysteine. This inhibition was reversed by concentrations of CdCl₂ greater than 0.25 mM; maximum activation in the absence of cysteine occurred at 0.5 mM CdCl₂. On the other hand, 1 mM solutions of L-cystine and L-leucine did not inhibit serum carnosinase.

Kinetics of L-leucine inhibition

Double-reciprocal plots of carnosinase activity and substrate levels at different concentrations of leucine (Fig. 18) show that the inhibition was complex. At low concentrations, leucine appeared to inhibit predominantly by a competitive mechanism, but at the higher concentrations, the inhibition shifted toward a noncompetitive mechanism. Fig. 19 shows the data plotted as reciprocal velocity vs. inhibitor concentration, the method of Dixon (1953).

The IC₅₀ of tissue carnosinase inhibition by L-leucine was 0.20 mM. The preparation employed in the kinetic assays contained two forms, which were separable by chromatography on Mono-Q (Chapter III). Therefore, it was possible that the pattern of plots in Fig. 18 were the result of a differential inhibition between the two carnosinase forms. To test this hypothesis, each form was individually assayed with varying leucine levels. The plots of percent inhibition of the two forms vs. leucine concentration were completely congruent, giving the same IC₅₀ value of 0.20 mM.
Fig. 18. Effects of L-leucine concentration and substrate concentration on carnosinase velocity. Double-reciprocal plots of velocity and substrate concentration at 0.8 (●), 0.4 (○), 0.2 (▲), 0.1 (□), and 0 (■) mM L-leucine. Each data point is the average of duplicate analyses. Regression lines are derived from computer-generated best fit hyperbolas.
Fig. 19. L-leucine inhibition of tissue carnosinase. Plots of reciprocal velocity (1/v) vs. L-leucine concentration (i) at substrate concentrations of 5 (▼), 10 (▲), 20 (★), and 40 (○) mM. Data are the same as in Fig. 18.
DISCUSSION

An edogenous inhibitor of human tissue carnosinase has been isolated and identified as L-leucine. When other common amino acids were also assayed for inhibitory activity, L-leucine was found to be most potent (Table VII). Evidently, one requirement for inhibition by an amino acid is an L-stereoconfiguration. L-leucine strongly inhibited carnosinase (87%), whereas D-leucine was essentially noninhibitory (5%). Racemic isoleucine and alanine were about half as active as their L-isomers. The most effective inhibitors were generally the more hydrophobic amino acids; L-leucine was much more inhibitory than glycine, L-tryptophan was more effective than L-tyrosine, and charged amino acids were not inhibitory. Amino acids having aliphatic side chains tended to be more potent than the aromatic amino acids; i.e., inhibition by L-leucine is greater than that by L-tryptophan.

Tissue carnosinase was also found to be inhibited by several dipeptides (Table VIII), some of which contained leucine. L-leu-L-leu inhibited carnosinase by 85%, whereas L-leucine inhibited by 74%; this suggests that L-leucine may bind to both R-group sites of the dipeptidase. The low inhibition by β-alanyl-DL-leucine (34%) was probably due to the racemic mixture of leucine. It should also be noted
that the measured inhibition by the dipeptides are probably minimum values; their concentrations may have decreased during the assay because of hydrolysis by carnosinase or by other peptidases present in the digest.

If some of the inhibitory dipeptides were present in the liver extract, they may have significantly contributed to the overall inhibitory potency of the extract. The sulfosalicylic acid supernatant contained about 3/4 of the original liver extract activity. Furthermore, the inhibitors are dialyzable, thermostable over a wide pH range, and pass unadsorbed through DEAE- and CM-cellulose; these results would be expected for inhibitory dipeptides or other small peptides. At least four unidentified ninhydrin-positive peaks were noted on the tracing from the amino acid analyzer between the peaks of Phe and Lys. Many dipeptides containing hydrophobic side chains have been reported to elute in that region from ion-exchange chromatography, including ala-leu, gly-leu, ala-norleu, ala-norval, gly-norleu, and gly-norval (Hamilton, 1963). Thus, much of the inhibitory activity contained in liver extracts may have been inhibitory dipeptides or oligopeptides which were hydrolyzed during the course of purification. Such hydrolysis could have resulted from microbial growth; no preservative was added to the extract, and several days had sometimes elapsed between certain steps of purification. However, no visible contamination was observed at any step.
Some of the inhibitory activity of the liver extract had eluted from the strong anion-exchange column (Fig. 13) and the silica gel column (Fig. 15), but were not further purified and identified. In addition, some of the inhibitory activity may have bound with high affinity to one of the columns without eluting from it.

Inhibition by L-leucine was found to be partially dependent on Mn$^{2+}$ (Fig. 17). This is similar to rat liver glycylglycine dipeptidase (Wilcox and Fried, 1963) which also shows greater inhibition by L-leucine in the presence than absence of the activating metal ion (Co$^{2+}$). This implies that L-leucine interacts with tissue carnosinase at the active site metal. Strong inhibition by L-leucine hydroxamate and the stereospecificity for L-leucine further support the theory of active site interaction.

Inhibition of tissue carnosinase by L-cysteine was reversed as the MnCl$_2$ concentration was increased above 0.02 mM. Cysteine is a strong chelator of metal ions, especially at alkaline pH (Gergely and Sovago, 1979) and its inhibition of carnosinase is probably caused by removal of the metal at the active site of this metalloexopeptidase. This mechanism of action would also account for tissue carnosinase inhibition by L-cystine, which is readily reduced to the cysteine monomer by the 2 mM dithiothreitol present in the digest (Cornell and Crivaro, 1972). Although serum
carnosinase was inhibited by L-cysteine, it was not by L-cystine. This enzyme is incubated without a reducing agent; hence, the dimer was not converted into L-cysteine. Cysteine inhibition of other dipeptidases and aminopeptidases has also been reported (Sugiura et al., 1977, 1978; Ito et al., 1983).

Plots of reciprocal tissue carnosinase velocity vs. L-leucine concentration (Dixon plots) demonstrated non-linear inhibition kinetics (Fig. 19). Since the lines are concave upward, the usual methods for $K_i$ determination, which require linear inhibition kinetics, may not be employed. Determination of the true inhibitor constant(s) depends upon the mechanism of inhibition. The curvilinear shape of the plots in Fig. 19 indicates that more than one molecule of inhibitor is able to combine with a single form of the enzyme (Dixon and Webb, 1979, p. 358). Furthermore, the pattern of double-reciprocal plots in Fig. 18 suggests that one molecule binds competitively and another binds with lower affinity noncompetitively. This would be similar to the L-leucine inhibition of a porcine kidney aminopeptidase (Wachmuth et al., 1966); L-leucine or other L-amino acids at low concentrations inhibited the aminopeptidase competitively but when the concentration was increased above that which caused a doubling of the $K_m$, the inhibition became mixed competitive and uncompetitive. Such an inhibition pattern with human tissue carnosinase was found not to be caused by
a differential inhibition of the two carnosinase forms, which were separable by Mono-Q chromatography but together during the kinetic study.

Human tissue carnosinase is inhibited by concentrations of L-leucine which are normally found in many tissues. The content of free leucine in rat brain and spleen has been reported to be 0.106 and 0.566 mmoles/kg wet weight, respectively, with most other organs containing intermediate leucine levels (Herbert et al., 1966). This is in rough agreement with the inhibitor content of various human tissues; spleen was most inhibitory and brain was least (Table VI). The correlation between free leucine content of rat tissues and percent carnosinase inhibition by human tissues was significant at $p<0.05$, in spite of the finding that only one fourth of the inhibitory activity of liver was attributed to inhibitory amino acids. Presumably, tissues having high levels of leucine may also have greater amounts of leucine-containing dipeptides than tissue of a lower free leucine concentration. Among biopsied human tissues, leucine levels were reported as 0.15 mM in the intracellular water for skeletal muscle (Bergstrom et al., 1974; Vinnars et al., 1975) and 0.07 μmoles/g wet wt. for cerebral cortex (Perry et al., 1971b). Tissues obtained at autopsy may contain quite different levels of free amino acids than biopsy samples; for example, the concentration of leucine in autopsied cerebral cortex was 0.32 μmoles/g (Perry et al., 1971a&b).
Leucine has been implicated as a regulator of protein turnover in the liver (Grinde and Seglen, 1981; Poso et al., 1982), skeletal muscle (Buse and Reid, 1975; Fulks et al., 1975; Hong and Layman, 1984), cardiac muscle (Chua et al., 1979), and macrophages (Besterman et al., 1983). L-leucine and, to a lesser extent, other branched-chain amino acids were generally found to inhibit protein degradation and stimulate protein synthesis. Although leucine application to muscles of starved rats enhances protein synthesis without influence on protein degradation (Hong and Layman, 1984), the predominant effect of leucine upon tissues of nourished animal tissues appears to be the inhibition of protein degradation (Seglen et al., 1980). L-leucine inhibits an unspecified aminopeptidase (Wachsmuth et al., 1966) and leucine aminopeptidase (Hill and Smith, 1956) from hog kidney, alanine aminopeptidase from human liver (Garner and Behal, 1975), peptidases from Calliphora flies (Collett, 1976), and glycyglycine dipeptidase from rat liver (Wilcox and Fried, 1963). For these enzymes the amount of leucine needed for 50% inhibition was greater than 0.2 mM, the IC₅₀ for carnosinase, but some similarities to leucine inhibition of human tissue carnosinase may be seen, including the mixed competitive-noncompetitive mechanism of inhibition (Wachsmuth et al., 1966) and the maximal inhibition in the presence of the activating metal ion
(Wilcox and Fried, 1963). Thus, leucine exerts a peptide-sparing effect on tissues by inhibiting peptidases, including tissue carnosinase.
CHAPTER V
BESTATIN INHIBITION OF TISSUE CARNOSINASE

INTRODUCTION

Bestatin is a dipeptide isolated from culture filtrates of *Streptomyces olivoreticuli* by Umezawa and coworkers (1975) in their search for enzyme inhibitors of therapeutic value. It was found to be a very potent competitive inhibitor of leucine aminopeptidase (EC 3.4.11.1) and aminopeptidase B (EC 3.4.11.6) with $K_i$ values of 20 nM and 60 nM, respectively (Suda et al., 1976), but was ineffective against a large number of other exopeptidases and endopeptidases (Aoyagi et al., 1977).

Clinically, bestatin is a potentially useful immunostimulant and chemotherapeutic agent. Bestatin enhances cell-mediated immunity *in vitro* (Ishizuka et al., 1980b; Blomgren and Wasserman, 1981) and *in vivo* (Umezawa et al., 1976; Ishizuka et al., 1980a). It is able to restore delayed-type hypersensitivity impaired by anticancer agents in mice (Ishizuka et al., 1980a) and in patients (Blomgren et al., 1980; Noma et al., 1981). By itself, bestatin also has antitumor activity (Tobe et al., 1980; Tsururo et al., 1981) and has been reported to be clinically effective against urogenital malignancies (Akiya and Okabe, 1981; Yokoyama et al., 1981). In addition,
bestatin increases the resistance of mice to infection by bacteria (Harada et al., 1983; Dickneite et al., 1984).

The mode of action of bestatin on the molecular level is not clearly known. The agent binds to and inhibits aminopeptidases on the surface of cells (Suda et al., 1976), especially macrophages (Leyhausen et al., 1983). It is reported to promote the synthesis and release of mitogenic factors, such as interleukin 1 and 2 (Blomgren et al., 1981; Umezawa and Aoyagi, 1983). It also acts on T-cells to induce DNA polymerase A activity, stimulating DNA and RNA synthesis and polysome assembly (Muller et al., 1979, 1981).

Recently, Margolis and coworkers have mentioned (without presenting data) that the Mn-dependent carnosinase from mouse kidney was inhibited by bestatin (Margolis et al., 1983; Margolis and Grillo, 1984). Since bestatin is a well known inhibitor of certain aminopeptidases, they concluded that this carnosinase is probably an aminopeptidase, not a true dipeptidase. Human tissue carnosinase is a dipeptidase (Chapter III), distinct from leucine aminopeptidase and aminopeptidase B.

In this chapter bestatin is reported to be an extremely potent competitive inhibitor of human tissue carnosinase ($K_i = 5.0 \times 10^{-10} \text{M}$). The characteristics of the inhibition further distinguish tissue carnosinase from other bestatin-sensitive enzymes.
MATERIALS AND METHODS

Materials

Bestatin ([(2S,3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine) (molecular weight, 308.2) was kindly provided as a powder by Professor H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). Human pancreatic dipeptidase was generously given by Dr. M. Sugiura (Gifu College of Pharmacy, Gifu, Japan). Cytosolic and microsomal porcine kidney leucine aminopeptidases were purchased from Sigma Chemical Co. (St. Louis, Missouri). Human brain carnosinase was partially purified by chromatography on DEAE-cellulose as described in Chapter III. Human serum carnosinase and hog kidney carnosinase were similarly prepared. Hog kidney homocarnosinase was partially purified by chromatography on DEAE-cellulose and Sephadex G-75 columns.

Enzyme and inhibitor assays

All assays using human tissue and plasma carnosinases, human pancreatic dipeptidase, and hog kidney homocarnosinase were performed as described in Chapters II and III. Assay conditions for cytosolic and microsomal leucine aminopeptidases followed those of Leyhausen et al. (1983).
RESULTS

**Effect of bestatin concentration on carnosinase activity**

Bestatin at extremely low concentrations inhibited carnosine hydrolysis by tissue carnosinase (Fig. 20); the IC$_{50}$ from 5 determinations was 4.2 ± 0.9 (s.d.) nM. This is smaller than the IC$_{50}$ of bestatin against leucine aminopeptidase (32 nM) and aminopeptidase B (160 nM) (Suda et al., 1976).

Since bestatin is a dipeptide, the possibility that it is hydrolyzed by carnosinase was investigated. The degree of inhibition by 4 nM bestatin did not decrease during the time of incubation with substrate for up to 95 min. Thus, bestatin appears not to be a substrate of carnosinase. Muller et al. (1982) also did not detect degradation products of bestatin after incubation with whole mouse lymphoma cells.

**Bestatin inhibition of other enzymes**

Bestatin is a more potent inhibitor of human tissue carnosinase than of other tested enzymes (Table IX). Human plasma carnosinase and hog kidney homocarnosinase were inhibited only by rather high concentrations of bestatin; these two enzymes also have similar optimum conditions for homocarnosine hydrolysis (Chapter II). The cytosolic form of
Fig. 20. Inhibition of tissue carnosinase by bestatin. Human kidney (○) and brain (+) partially purified carnosinase preparations were assayed in the presence of various bestatin concentrations. Each point is a single determination.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human tissue carnosinase</td>
<td>$4.2 \times 10^{-9}$</td>
</tr>
<tr>
<td>Human plasma carnosinase</td>
<td>$2.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Hog kidney carnosinase</td>
<td>$4.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Hog kidney homocarnosinase</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Leucine aminopeptidase, cytosolic</td>
<td>$1.4 \times 10^{-8}$</td>
</tr>
</tbody>
</table>
leucine aminopeptidase was much more sensitive to bestatin than was the microsomal form, which was not inhibited by 0.2 μM; this agrees with the literature (Leyhausen et al., 1983). Human pancreatic dipeptidase, a "true" dipeptidase (Ito et al., 1983) was only slightly inhibited by 3 μM bestatin. In addition to these results, a human liver dipeptidase (Sugiura et al., 1977) was found to be approximately 90% inhibited by 3 μM bestatin.

**Kinetics of bestatin inhibition**

Tissue carnosinase activity was measured under conditions of varied substrate and inhibitor concentrations. Fig. 21 shows the results plotted on double-reciprocal coordinants. The pattern is similar to what is expected for a tight-binding inhibitor where the enzyme and inhibitor are present at comparable concentrations (Morrison, 1969). In Fig. 22 the reciprocal velocity was plotted against the inhibitor concentration (Dixon, 1953); a concave upward curve was generated for each initial substrate level, rather than the more commonly reported linear Dixon plots. The experiment was repeated three times with similar results each time. This upward curvature also is characteristic of a tight-binding inhibitor (Henderson, 1972), where the usual assumption of total inhibitor concentration approximating unbound (free) inhibitor concentration is not valid.

To verify that bestatin does not irreversibly bind to carnosinase, a concentrated enzyme solution was pre-incubated
Fig. 21. Double-reciprocal plot of carnosinase activity vs. substrate concentration at 0 (O), 2 (□), 4 (○), and 6 nM (●) total bestatin. Each point is the average of two determinations, and the lines are derived from best-fit hyperbolas by least-squares analysis.
Fig. 22. Plots of reciprocal carnosinase velocity vs. total bestatin concentration. Initial substrate concentrations were: 5 (○), 10 (●), 20 (●), and 40 mM (●) carnosine. Data are the same shown in Fig. 21.
with 200 nM bestatin for 15 min and was then diluted to an assay concentration of 4 nM. The degree of inhibition was identical with the inhibition of the 4 nM bestatin control; i.e., the inhibition was completely reversible. Also, when carnosinase was diluted to one fifth of the standard assay concentration, it was much more strongly inhibited (81%) by 4 nM bestatin than was the standard concentration of enzyme (41%). Therefore, the amount of bestatin bound to carnosinase in the usual assay is a large proportion of the total inhibitor; the free bestatin concentration in equilibrium with the binding sites is considerably less than the total.

The concentration of free inhibitor \( I_f \) was calculated by subtracting the amount bound to the enzyme from the total bestatin concentration \( I_t \) according to the equation,

\[
I_f = I_t - \left[ e_t \left( \% \text{inhib} \right) / 100 \right]
\]

where \( e_t \) is the total enzyme concentration. The enzyme concentration was first estimated from Fig. 20 by a method of Dixon (1972) to be about 3.65 nM, but was later adjusted to 3.3 nM based on the best linear fit of the data. Fig. 23 shows that Dixon plots using free bestatin concentrations were linear and had a common intercept at \( K_i = 0.48 \) nM. The pattern is typical of either competitive or mixed competitive-noncompetitive inhibition.

To ascertain the type of inhibition, the computer-generated best-fit hyperbolas of the original data
Fig. 23. Inhibition kinetics of tissue carnosinase as a function of free bestatin concentration. Initial substrate concentrations were: 5 (○), 10 (●), 20 (●), and 40 mM (●) carnosine. The total enzyme concentration was assumed to be 3.3 nM based on the best linear fit of the data. The data are the same shown in Fig. 21.
(Michaelis-Menton plot) were employed to derive theoretical enzyme velocities at different total bestatin and substrate concentrations. These values, in turn, were used to generate Fig. 24 by the method of Henderson (1972). The intercept average, 3.38 nM, represents the total enzyme concentration. The slopes plotted against the substrate concentrations (Fig. 24, inset) show a linear positive correlation, demonstrating that the inhibition is predominantly, if not entirely, competitive.

**Effect of Mn$^{2+}$ on inhibition by bestatin**

Tissue carnosinase activity in the presence of 4 nM bestatin at various MnCl$_2$ concentrations was compared to controls without bestatin. Fig. 25A shows the activities with and without bestatin; no shift of optimum Mn$^{2+}$ concentration occurs. In Fig. 25B, the percent inhibition is plotted at each Mn$^{2+}$ concentration. The maximum degree of inhibition occurs in the same general area as the optimum Mn$^{2+}$ concentration.

**DISCUSSION**

Bestatin is an extremely potent competitive inhibitor of human tissue carnosinase. Table X compares the reported K values against different peptidases. Bestatin binds to
Fig. 24. Analysis of bestatin inhibition of tissue carnosinase by the method of Henderson (1972) for a tight-binding inhibitor. The best-fit hyperbolas from the original data were used to derive theoretical values of initial velocities at various substrate and total bestatin concentrations. The four resultant lines represent data at four initial carnosine concentrations (40, 20, 10 and 5 mM). \( I_t \) = total bestatin, \( v_i \) = velocity of reaction in the presence of inhibitor, and \( v_o \) = velocity without inhibitor. When the slopes of the lines were plotted against the initial substrate level (inset), the \( K_i \) of bestatin inhibition of carnosinase (y-intercept) was 0.5 nM. The linear positive correlation demonstrates competitive inhibition.
Fig. 25. Influence of MnCl$_2$ on tissue carnosinase inhibition by bestatin.

**A.** Effect of MnCl$_2$ concentration on carnosinase activity with bestatin at a total concentration of 4 nM (O) and without bestatin (Ø). Each point is the average of two determinations. The arrow designates the MnCl$_2$ concentration in the standard assay procedure.

**B.** Percent inhibition of tissue carnosinase as a function of MnCl$_2$ concentration. Percent inhibition at each MnCl$_2$ concentration is the decrease between control and inhibitor enzyme velocities divided by the control velocity.
A

Carnosine hydrolyzed (umoles)

MnCl₂ concentration (M)

B

% Inhibition

MnCl₂ concentration (M)
# TABLE X

REPORTED $K_i$ VALUES OF BESTATIN-INHIBITED ENZYMES

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue carnosinase</td>
<td>0.5</td>
<td>---</td>
</tr>
<tr>
<td>Leu-aminopeptidase, cytosolic</td>
<td>9</td>
<td>a</td>
</tr>
<tr>
<td>Leu-aminopeptidase</td>
<td>20</td>
<td>b</td>
</tr>
<tr>
<td>Dipeptidase, bacterial</td>
<td>30</td>
<td>c</td>
</tr>
<tr>
<td>Aminopeptidase-B</td>
<td>60</td>
<td>b</td>
</tr>
<tr>
<td>Leu-aminopeptidase, microsomal</td>
<td>3030</td>
<td>a</td>
</tr>
<tr>
<td>Aminopeptidase-M</td>
<td>4100</td>
<td>d</td>
</tr>
</tbody>
</table>

a (Leyhausen et al., 1983)  b (Suda et al., 1976)  
c (Hwang et al., 1982)  d (Rich et al., 1984)
carnosinase with 18 to 40 times greater affinity than to nonparticulate leucine aminopeptidase and with 120 times greater affinity than to aminopeptidase B.

This raises the question of whether carnosinase could be the pharmacological receptor of bestatin in its action as an immunostimulant. The binding of bestatin to mouse lymphoma cells was reported to have an association constant of $0.8 \times 10^{-5} \text{ M}^{-1}$ (Leyhausen et al., 1983), which is comparable to $K_i = 1.2 \times 10^{-5} \text{ M}$. However, concentrations as low as 32 nM were reported to stimulate incorporation of [3H]thymidine into lymphocytes of a mouse spleen cell culture (Ishizuka et al., 1980b). Therefore, some of the pharmacological action of bestatin may occur at a concentration well below saturation of total binding sites.

Based on the molar activity of carnosinase and its activity in the human spleen (Table III, p.65), and assuming that 1/4 of the total spleen weight is intracellular fluid, carnosinase concentration in cytosol would average $7.2 \times 10^{-7} \text{ M}$. If a typical cell has a volume of about $1.5 \times 10^3 \text{ cu \mu m}$, as in mouse lymphoma cells (Muller et al., 1982), there would be approximately $1.6 \times 10^5$ molecules of carnosinase per cell. Muller et al. (1982) found that mouse lymphoma cells had a total binding capacity of $14 \times 10^5$ molecules per cell. Thus, any binding to carnosinase may have been masked by the total cell binding.

For carnosinase to be the site of pharmacological action by bestatin, it must be accessible to the drug.
Bestatin binds to outer membrane aminopeptidases and has been assumed not to enter into the cell, based primarily on the work of Aoyagi et al. (1978) and Muller et al. (1982). Human tissue carnosinase appears to be a cellular enzyme; there was no indication of membrane-bound activity during fractionation experiments (Chapter III). However, Margolis and coworkers (Margolis et al., 1983; Margolis and Grillo, 1984) mention (without presenting data) that the Mn-dependent carnosinase of mouse tissues is partially membrane-associated. If this were true in human tissues, then carnosinase might be present on the outer cell membrane surface and available for bestatin binding. Another possibility is that bestatin may be able to enter the cell by a dipeptide transport system. Bestatin uptake into isolated epithelial cells from rat intestine has been indirectly demonstrated by Yasumoto and Sugiyama (1980); intracellular hydrolysis of glycyl-L-leucine was inhibited by extracellular application of 0.1 mM bestatin. At this concentration bestatin has essentially no effect on the uptake of carnosine (Yasumoto and Sugiyama, 1980) or glycylglycine (Himukai et al., 1982) into intestinal cells. At this point, a molecular mechanism by which carnosinase inhibition could stimulate cell-mediated immunity is completely unknown. Whether carnosinase is the pharmacological site of action for bestatin remains an open question and awaits further investigation.
Human tissue carnosinase is a dipeptidase (Chapter III) and is different from leucine aminopeptidase in its ability to split carnosine and inability to hydrolyze tripeptides (Smith and Spackman, 1955), as well as its apparent molecular weight (Hanson et al., 1965; Himmelhoch, 1969). Yet the two peptidases have much in common; both are best stabilized and activated by Mn$^{2+}$, completely inhibited by EDTA, maximally active at pH values greater than 9.0, able to hydrolyze L-histidylglycine and glycyl-L-leucine (Smith and Spackman, 1955), and inhibited by bestatin (Umezawa et al. 1975) and by hydrophobic branched-chain amino acids (Hill and Smith, 1957). In view of the similarities in their catalytic properties, it is probable that the mechanism of hydrolysis by both enzymes is very similar. Therefore, the proposed mechanisms by which bestatin inhibits leucine aminopeptidase should be valid for tissue carnosinase as well.

Fig. 26 compares the structures of bestatin and carnosine. These dipeptides have identical backbone structures, differing only in their side chains. It is interesting to note the presence of leucine, shown in Chapter IV to be the most potent of the common amino acid inhibitors of tissue carnosinase.

Two schematic models for the binding of bestatin to aminopeptidases have been proposed. In one scheme (Nishizawa et al., 1977), the hydroxyl group on carbon #2 and
Fig. 26. Structures of bestatin and carnosine.
free amino group of bestatin are chelated to the active-site metal. In the other (Nishino and Powers, 1979), the hydroxyl group and the carbonyl group are the metal ligands. In either case, bestatin is thought to be an analog of the transition state or tetrahedral intermediate for peptide bond hydrolysis (Ricci et al., 1982; Rich et al., 1984). Both proposed mechanism involves chelation with the activating metal. Bestatin inhibition of tissue carnosinase appears to be partially dependent on Mn$^{2+}$ concentration (Fig. 26). Any difference between carnosinase and leucine aminopeptidase in their affinities for bestatin is likely to be the result of differences in binding of the dipeptide R-groups and in the general "fit" of bestatin into the active site.

Bestatin and carnosine have identical backbone chains, different from common dipeptides. Carnosinase is also unusual, since the dipeptidase is able to splits a substrate containing beta-alanine; "ordinary" dipeptidases act only on substrates containing two alpha-amino acids (Das and Radhakrishnan, 1973). Therefore it is proposed that this "fit" of the bestatin backbone chain is an important component of its high affinity to tissue carnosinase.

This is not the first report of a dipeptidase being inhibited by bestatin. Hwang and coworkers (1981, 1982) have shown that it also inhibits an enzyme that predominantly splits dipeptides (Table X). This cobalt-activated enzyme
hydrolyzed gly-gly-gly at a rate only 2% that of the most active substrate. The dipeptidase was unable to split glycyl-L-leucine or glycyl-L-histidine, in contrast to carnosinase. Differences between these dipeptidases in affinity for bestatin may reflect different binding characteristics for chelation of the two metal ions as well as for the hydrophobic R groups. McIntyre and Curthoys (1982) reported that a dipeptidase from rat kidney was not inhibited by bestatin.

Carnosine is a putative neurotransmitter in the olfactory bulb of the mouse (Rochel and Margolis, 1982). Carnosinase may be the mechanism of inactivation of carnosine at the synapse; Rochel and Margolis (1982) were unable to demonstrate a high affinity carnosine uptake system in synaptosomal membrane preparations. Carnosinase activity in the olfactory bulb is associated with the olfactory nerve, since axotomy substantially reduced carnosine hydrolysis (Harding et al., 1977). There are two forms of carnosinase in mouse tissues (Margolis et al., 1979; Margolis and Grillo, 1984). The Mn-independent form is not present in the olfactory bulb (Margolis et al., 1983); whereas the other form, found in CNS tissues (Margolis et al., 1979), is Mn-dependent and has been reported (without presented data) to be inhibited by bestatin (Margolis et al., 1983; Margolis and Grillo, 1984). Apparently, the Mn-dependent form is responsible for
carnosine hydrolysis in the olfactory bulb. It is very likely that this carnosinase is homologous to human tissue carnosinase, a dipeptidase (Chapter III), and is not a general aminopeptidase, as previously assumed (Margolis et al., 1983; Margolis and Grillo, 1984). Therefore, the olfactory pathway probably contains the specific enzyme, tissue carnosinase, which degrades synaptically released carnosine. Further purification and immunohistochemical localization of this dipeptidase in the olfactory bulb are needed to establish whether carnosinase plays an important role at primary olfactory synapses. Presumably, bestatin-coupled affinity chromatography would facilitate the purification of tissue carnosinase. Bestatin may also be a useful tool in neurophysiological studies on the olfactory pathway.
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