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ENZYMATIC AND IMMUNOLOGICAL EVIDENCE FOR TWO FORMS OF CARNOSINASE IN THE MOUSE

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Summary

Carnosinase (aminoacyl-L-histidine hydrolase, EC 3.4.13.3) hydrolyzes the dipeptide carnosine (β-alanyl-L-histidine), which is thought to play a role in cerebral and skeletal muscular function and has been implicated as a neuro-affector in the olfactory bulb. Carnosinase activity is present in many tissues of the mouse including heart, liver and lung, but it is most active in kidney, uterus and nasal olfactory mucosa.

Kinetic measurements with ^{1}H-NMR spectroscopy indicate that the enzyme is stereospecific and can hydrolyze L- but not D-carnosine. Anserine is a poorer substrate, while homocarnosine is essentially a non-substrate. However, these two dipeptides are effective inhibitors of the hydrolysis of L-carnosine. Carnosinase activity is unaffected when assayed in ^2H_2O at 99% isotopic purity.

From considerations of the effect of Mn^{2+} on (1) substrate concentration velocity curves; (2) thermostability, and (3) inhibitor behavior, tissues with carnosinase can be divided into two groups. Kidney, uterus and olfactory mucosa represent one group, while central nervous system, muscle, spleen, etc. represent the second. The validity of this classification is confirmed by immunological evidence. Antiserum prepared against carnosinase purified from kidney cross-reacts with and inhibits the activity of olfactory mucosa, kidney and uterus but not that from central nervous system, heart or liver.

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Introduction

Imidazolyl dipeptides are unevenly distributed in the tissues of many species [1—4] and are generally present at high levels in excitable tissues. The physiological role of the imidazolyl dipeptides in muscle remains an enigma [5,6] but has been implicated to play a role in ion movements [7]. Nervous tissue of mammals, birds and amphibia also contains imidazolyl dipeptides [8—15] and the level of carnosine (β-alanyl-L-histidine) is particularly high in the primary olfactory pathway [16—21]. A series of biochemical studies have indicated that carnosine is present within the olfactory chemoreceptor neurons and have led to the suggestion that this dipeptide may function in neural tissues as a neurotransmitter or modulator (for review see Ref. 23).

The synthetic and degradative enzyme activities associated with carnosine metabolism are selectively elevated in certain mammalian tissues. Thus, in rodents, carnosine synthetase activity is much higher in nasal olfactory mucosa and olfactory bulb [22,24,25] than in other brain regions or in skeletal muscle, and after cell separation it has been reported to be present in neuronal as opposed to glial cells [26]. Partial purification and characterization of this enzyme from rodent cerebral tissues has been reported [24,27].

Carnosinase (aminoacyl-L-histidine hydrolase, EC 3.4.13.3) activity occurs in many vertebrate species [22,28—33] including the olfactory tissue of the garfish and the salamander *Ambystoma tiginum* (unpublished observations). The levels of carnosinase activity measured in kidney and nasal olfactory epithelium are as much as 40 times higher than those seen in other tissues such as skeletal muscle, olfactory bulb and brain [22]. The occurrence of anserinase [35,36] and homocarnosinase [37] has also been reported in certain species. Murphey et al. [38,39] reported the existence of two forms of carnosinase in humans and the association of one of these two forms with mental retardation. More recently Wolos et al. [40] have also reported two forms of carnosinase activity in swine kidney.

In view of our interest in the role of carnosine in cerebral function, we began a study of carnosinase activity in mice to evaluate whether there are different forms of the enzyme activity in different tissues of this species.

Materials and Methods

Carnosinase activity measurement by a radiometric method. Tissues were dissected onto solid CO_2 and weighed. Extracts were prepared for the radiometric assay by homogenizing in a polytron tissue disruptor with 5 or 10 vols. of 0.1 M Tris-HCl, pH 8.0. The extracts were centrifuged at 100 000 × *g* for 60 min and the supernatant fluids dialyzed overnight in the cold against two changes of 0.1 M Tris-HCl, pH 8.0, in the presence or absence of 1 mM $MnCl_2$. Carnosinase activity in the dialyzed supernatants was estimated by monitoring the formation of β-[^{14}C]alanine, which results from the hydrolysis of L-[*β-alanyl*-1-^{14}C]carnosine, under conditions that gave linearity as a function of time and enzyme concentration. The reaction mixture contained, in 0.55 ml, appropriate dilutions of tissue extracts, 0.075 M Tris-HCl, pH 8.0, 1 mM aminooxyacetic acid, 250 000 cpm of ^{14}C-labeled carnosine plus unlabeled

carnosine and $MnCl_2$ to final concentrations as indicated in the tables and text. Aminooxyacetic acid was added to prevent degradation of the β-[1-^{14}C]alanine product by any β-alanine transaminase activity that might be present in the extract. After incubation at 37°C for 60 min the reaction was stopped by the addition of 1.0 ml of cold ethanol containing 1 mM β-alanine. After centrifugation in the cold, the supernatant was applied to a 2 cm × 0.6 cm column of Dowex 50X-8, 200—400 mesh resin in the α-picoline form [22]. The column was washed twice with 2 ml of H_2O and the eluates discarded. The β-[1-^{14}C]-alanine was eluted from the resin with 4 ml of 0.1 M α-picoline. 10 ml of Aquasol were then added and radioactivity determined in a refrigerated scintillation counter. The reaction blank was about 500 cpm and the enzymatic activity at least 5—10 times this.

Immunoenzymatic titrations. Mouse kidney carnosinase was purified to homogeneity and rabbit antisera were prepared (manuscript in preparation). Tissue extracts were prepared as above for the radiometric assay and undialyzed aliquots incubated for 30 min at 37°C with appropriate dilutions of antiserum or control serum in a final volume of 500 μl containing 80 mM Tris-HCl, pH 8.0, 1 mM aminooxyacetic acid and, if appropriate, 1 mM $MnCl_2$. A suspension of goat anti-rabbit IgG bound to kynar beads in 80 mM Tris-HCl, pH 8.0, was then added (200 μl) and incubation continued for an additional 15 min at 37°C. After centrifugation 500 μl of the supernatant was added to 50 μl of a solution containing 250 000 cpm of ^{14}C-labeled carnosine, plus unlabeled carnosine to give a final concentration of 200 μM. Carnosinase activity was then determined as above.

Carnosinase activity by ^{1}H-NMR spectroscopic method. Tissues obtained as above were homogenized with 1.5 vols. of 100 mM sodium phosphate buffer, pH 7.4, with a polytron tissue disruptor for twice 10 sec at setting 6. The supernatants obtained after centrifugation at 40 000 × *g* for 2 h were dialyzed twice in the cold against 50 vols. of the above buffer in ^2H_2O, adjusted to pH 7.4 with NaO^2H. pH values were not corrected for isotope effects. One-tenth volume of 100 mM dipeptide solution in ^2H_2O, adjusted to pH with NaO^2H or ^2HCl, was added to the soluble tissue preparation and incubated at 37°C. At various times 0.5 ml aliquots were transferred to NMR tubes containing 1 mg solid EDTA and held at 0—5°C. This completely stopped the hydrolysis until ^{1}H-NMR spectra could be recorded.

The ^{1}H-NMR spectra were obtained with pulse-Fourier transform techniques on a Varian XL-100 spectrometer at ambient probe temperature (approx. 30°C). 300 pulse-induced free induction decays were collected with a pulse interval of 4 s. All spectra covered a spectral range of 1000 Hz with a resolution of 0.25 Hz/channel. The concentrations of unhydrolyzed substrate and constituent amino acids resulting from hydrolysis were determined from the peak areas of resonances arising from each component of the mixture [41]. It was determined in a series of preliminary experiments that the carnosinase activities measured by the radiometric and ^{1}H-NMR assays were in very close agreement. It was also demonstrated that the substitution of 99.8% ^2H_2O for H_2O had no effect on the carnosinase activity as measured by the radiometric assay.

Materials. Retired breeder female albino mice (CD-1) were purchased from

Charles River Laboratories, North Wilmington, MA, and maintained with ad libitum access to food and water. Mice were killed by CO_2 asphyxiation followed by exsanguination. L-[*β-alanyl*-1-^{14}C]Carnosine (spec. act. = 9.7 Ci/mol) was custom synthesized by New England Nuclear, Boston, MA, from whom Aquasol was also purchased. The peptides used in the inhibitor study were from Sigma Chemical Co., St. Louis, MO, and Vega Fox, Tucson, AZ. All compounds identified with an RO code number were from the Chemical Research Department of Hoffmann-La Roche Inc., Basle, Switzerland. The Dowex, 50X-8, 200—400 mesh resin was from Biorad, Richmond, CA. ^2H_2O (99.8% isotopic purity), NaO^2H, ^2HCl and the NMR sample tubes were purchased from Wilmad Glass Co., Buena, NJ. Goat anti-rabbit IgG on kynar beads was supplied by Roche Diagnostics Division, Nutley, NJ.

Results

Kinetics of carnosinase

Carnosinase activity is present in many tissues (Table I) and is especially high in extracts of kidney, olfactory mucosa and uterus. Of the total activity in the initial homogenates of olfactory mucosa, kidney and central nervous system, 98, 95 and 95%, respectively, was recovered in the supernatant extract. The amount of activity observed is a complex function of both substrate and Mn^{2+} concentration. Thus, at 200 μM carnosine concentration, the addition of 1 mM Mn^{2+} inhibits the carnosinase activity of kidney, nasal olfactory mucosa and uterus, while it stimulates the carnosinase activity of other tissues. However, at 5 mM carnosine concentration, the addition of 1 mM Mn^{2+} stimulates the activity in all tissues but to different degrees. For kidney, olfactory mucosa and uterus the apparent increases are 1.5, two and four-fold, respectively, while for other tissues a 20—40-fold increase is observed (Table I). This relationship between Mn^{2+} and carnosine concentration is maintained after purification of

TABLE I

TISSUE DISTRIBUTION OF CARNOSINASE ACTIVITY AND EFFECT OF CARNOSINE CONCENTRATION ON Mn^{2+} STIMULATION

Dialyzed tissue extracts prepared as described in the text were assayed radiometrically at carnosine and $MnCl_2$ concentrations noted in the column headings.

Tissue	Carnosinase activity (nmol/h per mg tissue)			
Carnosine (μM)	200	200	5000	5000
$MnCl_2$ (mM)	—	1	—	1
Kidney	47.7	18.3	72.8	102.3
Olfactory mucosa	12.2	8.2	18.9	37.4
Uterus	6.3	2.6	9.0	35.1
Central nervous system	0.15	0.53	0.34	6.8
Liver	0.07	0.27	0.08	4.9
Lung	0.01	0.19	0.11	3.6
Heart	0.01	0.18	0.10	3.5
Muscle	0.02	0.12	0.14	2.2
Spleen	0.002	0.06	0.03	1.3

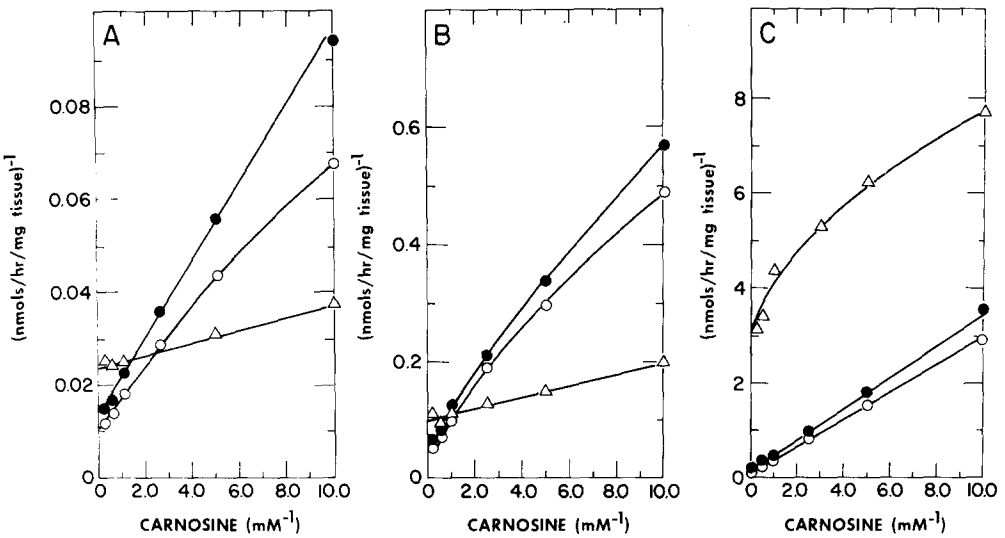

Fig. 1. Effect of Mn^{2+} on carnosinase activity determined radiometrically as a function of substrate concentration. Dialyzed extracts of kidney (A), olfactory mucosa (B) and central nervous system (C) were prepared as described in the text, and the carnosinase activities were assayed at various substrate concentrations (0.1—10 mM) and in the presence of $MnCl_2$ concentrations of 1 mM (○), 3 mM (●) or in its absence (△).

the kidney enzyme or ammonium sulfate fractionation of extracts of central nervous system or mucosa (data not shown).

These data suggest that substrate and metal ion interactions differ among the carnosinase activities in extracts of different tissues. Therefore, the effect of varying the Mn^{2+} and carnosine concentrations on the carnosinase activity in extracts from kidney, olfactory mucosa and central nervous system was evaluated. The similarity of response of the carnosinase activity in kidney and olfactory mucosa is evident from Fig. 1. In the absence of added Mn^{2+}, carnosinase activity of kidney and olfactory mucosa manifests apparent K_m values of 70 and 100 μM, respectively. Upon the addition of Mn^{2+}, the apparent K_m is at least ten-fold higher, the V appears to be doubled, and the lines now exhibit slight curvature (Fig. 1A and B). In contrast, carnosinase activity in the central nervous system is stimulated by Mn^{2+} at all carnosine concentrations with a 40-fold increase in V (Fig. 1C). In contrast to the results with mucosa and kidney extracts, curvature of the Lineweaver-Burk plot occurs with the central nervous system extracts in the absence, but not in the presence, of Mn^{2+}. These observations are consistent with the results in Table I. The reason for the observed curvature is not readily explained but may occur at least in part from the binding of Mn^{2+} by carnosine in solution (K_{diss} = 37 mM) [42].

Thermostability

The effect of Mn^{2+} on the thermostability of carnosinase activity in extracts from various tissues was evaluated at 50°C. In the absence of added Mn^{2+}, carnosinase activity in dialyzed extracts from both central nervous system and

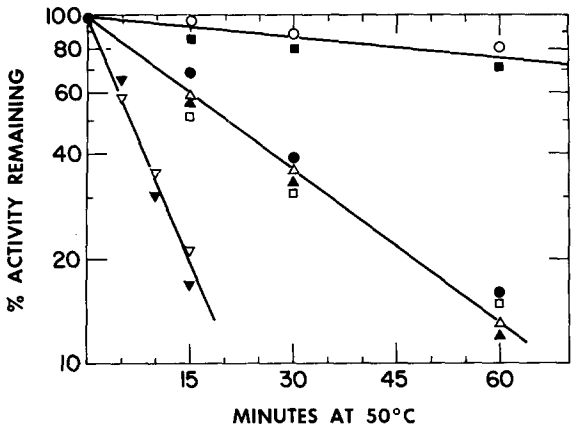

Fig. 2. Thermal inactivation profiles at 50°C for carnosinase activity of various mouse tissues. Soluble extracts were prepared at 20% (w/v) for muscle (△), central nervous system (●), spleen (□) and heart (▲) and at 10% (w/v) for olfactory mucosa (○) and kidney (■). After dialysis against two changes of 0.1 M Tris-HCl, pH 8.0, containing 1 mM $MnCl_2$ for all extracts or in the absence of $MnCl_2$ for kidney (▼) and central nervous system (▽), the extracts were incubated at 50°C. After various incubation times at 50°C, aliquots of the incubation mixtures were assayed radiometrically in the presence of 1 mM $MnCl_2$ and 200 μM carnosine as described in the text.

kidney is very unstable and exhibits a half-life of 6—8 min (Fig. 2). In the presence of Mn^{2+}, the $t_{1/2}$ is extended to 20 min for the activity from central nervous system, spleen, heart and skeletal muscle and to 150 min for the activity from mucosa and kidney (Fig. 2). Thus, the effect of Mn^{2+} on both thermostability and catalytic activity differentiates the tissues into two groups.

Substrate specificity

To gain some insight into the substrate specificity of the carnosinase activities, the rates of hydrolysis of L-carnosine, D-carnosine, L-anserine and L-homocarnosine by nasal olfactory mucosa, brain and kidney were measured by ^{1}H-NMR spectrometry. Carnosine hydrolysis by soluble extracts of olfactory mucosa produces a progessive change of the ^{1}H-NMR spectrum of the reaction mixture from that of the dipeptide carnosine to that of a mixture of the component amino acids histidine and β-alanine (Fig. 3). Thus, this technique permits direct simultaneous measurement of the hydrolysis of the pep-

TABLE II

SUBSTRATE SPECIFICITIES OF CARNOSINASE FROM VARIOUS TISSUES AS DETERMINED BY ^{1}H NMR SPECTROSCOPY

The values presented are specific activities expressed in nmol of substrate hydrolyzed/h per mg of tissue at 10 mM initial substrate concentration in the absence of exogenous Mn^{2+}. The lower limit of sensitivity of the assay is 0.2—0.4 nmol/h per mg. n.d., not detectable.

Substrate	Kidney	Nasal olfactory mucosa	Brain
L-Carnosine	143.0	20.9	0.62
D-Carnosine	≤0.20	n.d.	n.d.
Anserine	12.8	1.90	n.d.
Homocarnosine	≤0.39	n.d.	n.d.

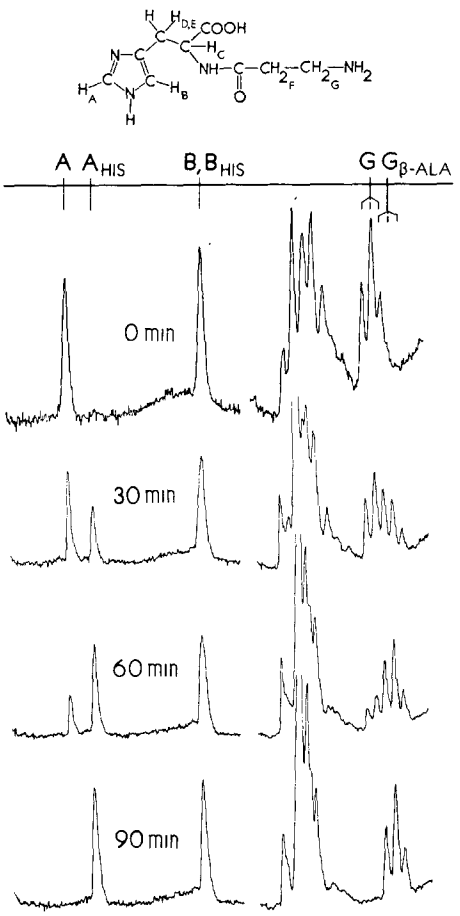

Fig. 3. ^{1}H-NMR spectra of a 40% soluble extract of nasal olfactory epithelium plus 10 mM L-carnosine after 0, 30, 60 and 90 min of incubation at 37°C. The left portion of each spectrum corresponds to the downfield aromatic region, and the right portion corresponds to the upfield aliphatic region. The large peak in the aliphatic region of the spectra recorded after 30, 60 and 90 min of incubation arises from the EDTA that was added to terminate the reaction. The concentration of carnosine in the reaction mixture is determined by the peak areas of the resonances of the C-2 proton of the histidinyl residue (labeled A) and of the β-methylene protons of the β-alanyl residue (labeled G). The concentrations of the component amino acids that were produced by hydrolysis of the peptide are determined from the peak areas of the resonances that arise from the analogous protons on the free amino acids (labelled A_H and $G_{\beta\text{-Ala}}$). The spectra were not printed out at the same spectrum amplitude, and the area of the peak labeled β,β_{His} is used as an internal reference.

tide and of the production of the component amino acids. The rates of hydrolysis of L-carnosine by kidney, olfactory mucosa and central nervous system at 10 mM initial substrate concentration in sodium phosphate buffer at pH 7.4 are linear (Fig. 4) and in the proportion 100 : 15 : 0.4, respectively (Table II). These data are in excellent agreement with the results in Table I, column 3, obtained with the radiometric assay. Anserine hydrolysis by olfactory mucosa extract is linear with time and is 0.09 times the rate observed for L-carnosine hydrolysis (Fig. 4 and Table II). Additionally, β-alanine and 1-methylhistidine or histidine increase equally and in exact proportion to the rate of dis-

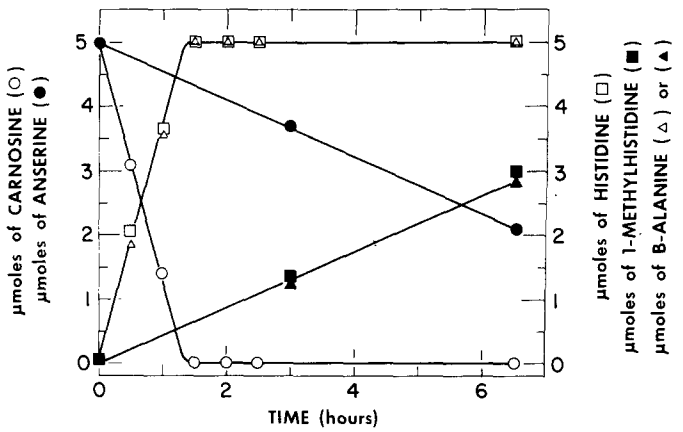

Fig. 4. Kinetics of hydrolysis of L-carnosine and L-anserine by a 40% soluble extract of nasal olfactory epithelium. The concentrations of the peptide substrates and the component amino acids were determined by measuring the peak areas of the ^{1}H-NMR spectra of the incubation mixtures at each time point (Fig. 3). The µmol of carnosine (○) and anserine (●) remaining at each time point are indicated. The histidine (□) and β-alanine (△) produced by hydrolysis of carnosine and the 1-methylhistidine (■) and β-alanine (▲) produced by hydrolysis of anserine are also represented.

appearance of the respective dipeptides (Fig. 4). Kidney extracts hydrolyzed L-carnosine, anserine, homocarnosine and D-carnosine in the ration 100 : 9 : <0.3 : <0.1, respectively (Table II). The hydrolysis of anserine, D-carnosine or homocarnosine by brain was not detectable nor was the hydrolysis of homocarnosine or D-carnosine by olfactory mucosa. Thus, in the absence of added Mn^{2+} the carnosinase activities from brain, kidney and nasal olfactory mucosa all exhibit stereospecificity and clear preference for L-carnosine as substrate.

Inhibitors

Several peptides were tested as potential inhibitors of carnosinase activity (Table III). Of the various substrate analogs tested, anserine and homocarnosine were the best inhibitors of carnosinase activity in all tissues and seem especially effective in the absence of added Mn^{2+}. The effectiveness of these dipeptides as inhibitors suggests that they have higher affinity for carnosinase than carnosine itself, even though they are poor substrates. D-Carnosine failed to inhibit the hydrolysis of L-carnosine even when the D-isomer was present at five times the concentration of the L-isomer. A five-fold increase in the concentration of L-carnosine results in increases of activity consistent with the data in Table I and Fig. 1. In the absence of added Mn^{2+} the other peptides tested had little or no effect as inhibitors of carnosinase activity in any of the tissues. However, in the presence of 1 mM Mn^{2+}, L-His-Gly, L-His-β-Ala and Gly-L-His-Gly stimulated carnosinase activity of kidney and olfactory mucosa but not that of brain and muscle. Several additional compounds were found to have no effect on carnosinase activity in any of these tissue extracts (Table III). However, one complex imidazole derivative inhibited the carnosinase activity of kidney, uterus and olfactory mucosa in the absence of 1 mM $MnCl_2$ and stimulated in its presence (Table IV). In contrast, this compound had no effect on the carnosinase activity from muscle, brain or heart.

TABLE III

EFFECT IF SUBSTRATE ANALOGS ON CARNOSINASE ACTIVITY IN SEVERAL TISSUES OF THE MOUSE

Dialyzed tissue extracts prepared as described in the text were assayed by the radiometric assay at 200 μM carnosine. Peptides were added at 1 mM final concentration. The following were inactive when tested on olfactory mucose and central nervous system at 1 mM final concentration: β-Ala-L-Ala, β-Ala-Gly, β-Ala-β-Ala, pyroglutamyl-L-histidinyl prolinamide (Ro 8-6270), *N*-acetylhistidine, diphenhydramine, pyrilamine maleate, cimetidine, metiamide, L-2-benzamido-3-[([5-methylimidazol-4-yl]methyl)thio]propionamide (Ro 12-4407) and 1-cyano-2-[2-imidazol-4-ylethyl]-3-methyl guanidine (Ro 0924).

Peptide	Caenosinase activity (nmol/h per mg tissue)							
	Kidney		Olfactory mucosa		Brain		Muscle	
$MnCl_2$ (mM)	—	1	—	1	—	1	—	1
None	52.6	16.7	10.8	4.7	0.18	0.56	0.016	0.13
D-Carnosine	52.1	16.6	11.0	4.7	0.18	0.54	0.017	0.013
L-Carnosine	69.4	58.0	13.3	14.8	0.27	2.7	0.05	0.69
Anserine	4.4	4.9	0.8	1.2	0.02	0.43	0.005	0.11
Homocarnosine	6.7	6.2	1.5	1.1	0.03	0.32	0.007	0.09
Glycyl-L-histidine	42.8	10.3	9.1	2.2	0.17	0.49	0.014	0.06
Glycyl-L-phenylalanine	46.0	11.1	10.1	3.2	0.19	0.28	0.015	0.05
L-Alanyl-L-histidine	42.7	21.0	9.3	5.5	0.18	0.44	0.013	0.03
L-Histidinyl-glycine	40.1	38.2	8.8	7.1	0.19	0.55	0.013	0.14
L-Histidinyl-β-alanine	37.7	37.4	8.6	6.8	0.17	0.48	0.014	0.12
Glycyl-L-histidinyl-glycine	41.4	29.8	9.3	6.6	0.18	0.57	0.014	0.13

Immunotitrations

Antiserum prepared against purified mouse kidney carnosinase partially inhibited the enzyme activity (data not shown) and in the presence of the second antibody eliminated virtually all of carnosinase activity from extracts of kidney, uterus or olfactory mucosa (Table V). The same quantity of antiserum had very little effect on extracts of central nervous system, spleen or liver. Additionally, it is evident that $MnCl_2$, while inhibiting the carnosinase activity of kidney, does not alter its ability to interact with the antiserum (Table V). We next evaluated the influence of increasing quantities of antiserum on the

TABLE IV

EFFECT OF Ro 12-3997 ON CARNOSINASE ACTIVITY OF VARIOUS TISSUES

Carnosinase activity was determined radiometrically as in Table III. Ro 12-3997 is (((L-1-(hydroxymethyl)-2-(imidazol-4-yl)ethylamino)-(methylamino)methylene)urea.

Tissue	Carnosinase activity (nmol/h per mg tissue)			
$MnCl_2$ (mM)	—	—	1	1
Ro 12-3997 (mM)	—	1	—	1
Muscle	0.02	0.02	0.17	0.17
Central nervous system	0.18	0.18	0.66	0.62
Heart	0.01	0.01	0.16	0.16
Kidney	69.1	34.4	34.9	50.4
Olfactory mucosa	15.4	10.0	9.0	10.1
Uterus	7.9	2.9	4.9	6.9

TABLE V

EFFECT OF MOUSE KIDNEY CARNOSINASE ANTISERUM ON CARNOSINASE ACTIVITY IN VARIOUS MOUSE TISSUES

Assays were performed in duplicate as described in Materials and Methods.

Tissue	$MnCl_2$ (mM)	Carnosine hydrolyzed (nmol)		
		No serum	Control serum (0.5 μl)	Immune serum (0.5 μl)
Kidney	—	14.6	13.4	0.2
Kidney	1	6.4	8.0	0.2
Olfactory mucosa	—	10.8	10.7	0.1
Uterus	—	7.3	9.0	0.1
Central nervous system	1	14.6	14.6	11.4
Liver	1	17.8	17.9	17.7
Spleen	1	30.4	31.0	30.4

carnosinase activity of extracts of central nervous system and kidney alone and in combination (Fig. 5). The influence on central nervous system was minimal while kidney was virtually totally inhibited. However, when the two were combined the antiserum inhibited the combined activity down to the level of

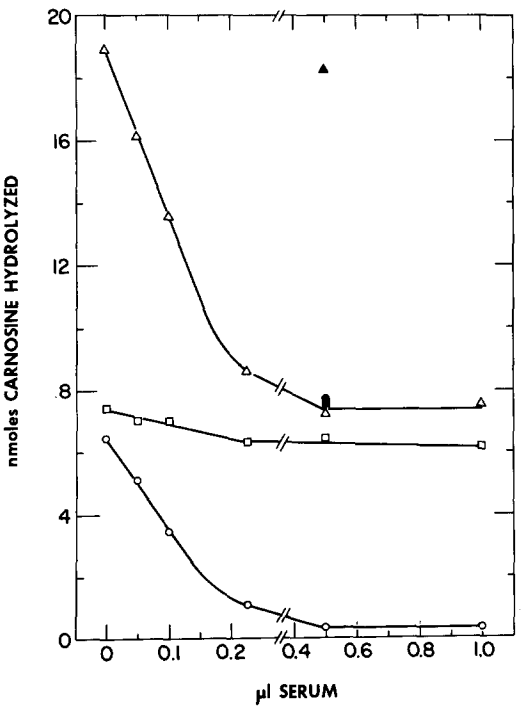

Fig. 5. Immunotitration of carnosinase activity of extracts from kidney and CNS alone and a mixture of the two. The extracts, at equal activities, either alone or in combination were preincubated in duplicate with increasing volumes of rabbit antiserum or control serum in the presence of 1 mM $MnCl_2$ and then with goat anti-rabbit IgG beads. After centrifugation the carnosinase activity remaining in the supernatant was assayed radiometrically. Kidney (○), central nervous system (□) and mixture (△) in the presence of antiserum. Kidney (●), central nervous system (■) and mixture (▲) in the presence of control rabbit serum.

the central nervous system alone, demonstrating the selectivity and specificity of this antiserum in the presence of a mixture of both enzymes.

Discussion

The carnosinase activity of olfactory mucosa, uterus and kidney is distinguishable from that of central nervous system, liver, lung, skeletal muscle, heart and spleen based on thermostability measurements, enzymological data and immunological criteria.

Enzymatically the most notable difference between these two groups of tissues is in the influence of Mn^{2+}. At 200 μM carnosine the carnosinase activity of kidney, nasal olfactory mucosa and uterus but not other tissues is inhibited by 1 mM $MnCl_2$. When the substrate concentration is raised to 5000 μM, the carnosinase activity of all tissues is stimulated by 1 mM $MnCl_2$ but central nervous system, lung, muscle, heart and spleen are an order of magnitude more responsive than mucosa, kidney and uterus. When the effect of Mn^{2+} on carnosine concentration curves was evaluated in detail, it was evident that the enzyme activity from central nervous system behaved very differently from that of olfactory mucosa and kidney. Mn^{2+} stimulation was seen at all concentrations of carnosine for central nervous system, while a cross-over was seen in kidney and mucosa at 0.5—1 mM carnosine concentration.

Determination of carnosine hydrolysis by various tissues will therefore vary as a function of the assay conditions. Thus, for example, the apparent capability of extracts from kidney to hydrolyze carnosine varies only six-fold, while that from central nervous system varied 45-fold under various conditions. Similarly, the differential between kidney and central nervous system can vary from 15 to 320-fold depending on the assay conditions. A wide variation in assay conditions exists among different laboratories studying carnosine activity. This fact may explain some of the differences in apparent K_m values and other parameters reported for these enzymes from various tissues of several species [22,25,31—40].

The presence of Mn^{2+} is essential for the thermal stability of carnosinase from all tissues, but it imparts a much greater stability to the carnosinase activity of kidney and nasal olfactory mucosa than it does to that of central nervous system, muscle, spleen and heart. The various tissues may be divided into two groups based on the effect of Mn^{2+} on both enzyme activity and thermal inactivation. Mucosa, kidney and uterus fall into one group, while muscle, central nervous system, spleen, heart, liver and lung fall into another group. Our data, thus, agree with previous reports that Mn^{2+} serves as both a stabilizer and activator of carnosinase [28—40] and suggest in addition that there are two different forms of this enzyme.

If there are two different forms of carnosinase activity then one might expect to observe differences in the recognition of substrates and substrate analogs. Therefore, we studied the hydrolysis of D- and L-carnosine, anserine and homocarnosine by extracts of kidney, olfactory mucosa and central nervous system. We utilized ^{1}H-NMR spectroscopy because this technique enabled us to measure simultaneously the kinetics of both the hydrolysis of substrate and the appearance of products. Carnosinase activity from all tissues

tested was absolutely stereospecific for the L-isomer of carnosine. Homocarnosine is not hydrolyzed, while in those tissues with high enough levels of carnosinase activity to permit detection (kidney and olfactory mucosa), anserine exhibited an intermediate rate of hydrolysis, as has been reported for carnosinase activity of other species [28,35,38]. Thus, in the absence of added Mn^{2+}, the substrate specificities seem quite similar in the different tissues.

However, when several carnosine analogs were tested as inhibitors of carnosinase activity, in the presence and absence of added Mn^{2+}, the enzymes from the different tissues were found to respond differently with some, but not all, analogs tested. As expected, D-carnosine, which is not a substrate, is also not an inhibitor of the carnosinase activity, further testifying to the stereospecificity of carnosinase. Anserine and homocarnosine, both of which occur naturally in biological systems but are poor substrates for carnosinase, are good inhibitors of, and have higher affinity for, both forms of the enzyme than does carnosine. Several peptide analogs of carnosine, as well as a complex imidazole derivative, behaved as modifiers of enzyme activity and were useful in discriminating among the tissues and helping to divide them into two groups.

If carnosinase activity is being expressed by two different proteins then antiserum prepared against one of these proteins should only react with the one, and not with the other, permitting an immunological demonstration of the existence of two distinct carnosinases. To test this, antiserum was generated against purified mouse kidney carnosinase and tested against various extracts. This antiserum totally eliminates the activity from extracts of kidney, olfactory mucosa and uterus but not from extracts of other tissues. This confirms the conclusion drawn from our enzymatic and thermostability studies that the carnosinase activity in different mouse tissues is due to the existence of two different proteins, each of which is capable of hydrolyzing carnosine. This is the first immunological evidence in support of reports of multiple forms of carnosinase activity in other species [37—40] and confirms that there are two structurally and functionally distinct forms of carnosinase in different tissues of the mouse.

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