Incorporation of [^{14}C]Histidine into Homocarnosine and Carnosine of Frog Brain *in vivo* and *in vitro*

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1. Homocarnosine, carnosine and histidine were determined in brain from several species and the results compared with values in the literature. 2. [^{14}C]Homocarnosine and [^{14}C]carnosine were isolated from frog brain after intracerebral injection of [^{14}C]histidine *in vivo*. 3. Whole frog brain, incubated *in vitro* with L-[^{14}C]histidine, formed labelled homocarnosine and carnosine. 4. A frog brain homogenate or supernatant fraction catalysed the incorporation of L-[^{14}C]histidine into homocarnosine and carnosine. 5. The results indicate that brain tissue can synthesize homocarnosine and carnosine.

Homocarnosine (γ -aminobutyryl-L-histidine) was identified by Pisano, Wilson, Cohen, Abraham & Udenfriend (1961) in bovine brain and isolated in crystalline form from the same source by Kanazawa, Kakimoto, Miyamoto & Sano (1965). Abraham, Pisano & Udenfriend (1962) found homocarnosine to be present exclusively in the central nervous system of pig, dog, cat and man. The same workers also reported the presence of small amounts of carnosine in brain tissue. In chick muscle, Kalyankar & Meister (1959) and Winnick & Winnick (1959) demonstrated the enzymic synthesis of carnosine and anserine. The enzyme system was rather non-specific and could catalyse the synthesis of many related dipeptides including homocarnosine (Kalyankar & Meister, 1959; Winnick & Winnick, 1958). Abraham, Pisano & Udenfriend (1961) found that homocarnosine could be isolated from muscle tissue when rats were fed on a diet containing large amounts of γ -aminobutyric acid, although brain homocarnosine concentrations did not change. The present paper reports experiments demonstrating the incorporation of [^{14}C]histidine into homocarnosine and carnosine by frog brain *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials. L-[ring-2- ^{14}C]Histidine was obtained from Calbiochem. Los Angeles, Calif., U.S.A.; the solution had radioactivity $50\mu\text{C}/\text{ml}$. and was used with a specific radioactivity of $35.0\mu\text{C}/\mu\text{mole}$ for the experiments *in vivo* and with a specific radioactivity of $10\mu\text{C}/\mu\text{mole}$ for the experiments *in vitro*.

Dowex AG-50 W (X4; 200–400 mesh; H^+ form) was obtained from Calbiochem. The silica-gel sheets used for t.l.c. were Eastman Chromagram Sheet no. 606 from Distillation Products Inc., Rochester, N.Y., U.S.A. Paper chromatography was performed with Whatman no. 1 paper. The Fast Red Salt TRN used in quantitative determination of histidine, carnosine and homocarnosine (see Abraham *et al.* 1962) was given by General Aniline and Film Corp., New York, N.Y., U.S.A.

Animals. Frogs (*Rana pipiens*; body length approx. 8 cm.) were supplied by E. G. Hoffman and Son, Oshkosh, Wis., U.S.A. The frogs were injected intracerebrally by inserting a no. 21 needle through the skull at a point midway between the eyes. The needle was held at a 60° angle and rotated slowly with pressure to puncture the dense tissue. As the skull was penetrated, the resistance greatly decreased; the insertion was halted at this point. Where penetration caused injury and bleeding, the frog was rejected. After the skull was penetrated, a micro-syringe (Hamilton Microliter no. 701; Hamilton Co. Inc., Whittier, Calif., U.S.A.) with needle was introduced through the bore of the larger needle. The micro-syringe needle was moved approx. 1.2 cm. beyond the point of the first needle into the cerebrum of the frog. This was also done at a slight angle to the midline of the frog to ensure penetration into the cerebrum. The plunger was slowly pushed into the micro-syringe, so that approx. 30 sec. was taken to empty it.

Experiments in vivo. Each frog received $10\mu\text{l}$. ($0.50\mu\text{C}$) of [^{14}C]histidine ($35\mu\text{C}/\mu\text{mole}$) injected intracerebrally for the test, or intraperitoneally for control studies. The experiment included two additional controls, one involving the intraperitoneal injection of three times the normal dose ($30\mu\text{l}/\text{frog}$; $1.5\mu\text{C}$) and one involving endocardial injection ($10\mu\text{l}/\text{frog}$; $0.5\mu\text{C}$) of [^{14}C]histidine.

After the injection of [^{14}C]histidine, frogs were decapitated after 1, 1.5 and 2 hr. The brains were immediately removed, weighed and homogenized in the cold in aq. 80% (v/v) ethanol. Four frog brains were prepared individually and then pooled. After centrifugation at 2000g for 10 min., the supernatants were extracted and treated as described by

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Abraham *et al.* (1962). Fractions from the Dowex 50 column corresponding to histidine, carnosine and homocarnosine were collected and concentrated. Samples were used for t.l.c. with radioautography, determination of radioactivity by liquid-scintillation methods, and quantitative determinations.

Experiments in vitro. The intact frog brain was immersed in a synthetic medium similar to the one used by Krebs (1950), containing (final concentrations) 124 mM-NaCl, 5 mM-KCl, 1.2 mM-KH₂PO₄, 1.3 mM-MgSO₄, 2.0 mM-CaCl₂, 25 mM-NaHCO₃ and 10 mM-glucose. To each tube containing 1.0 ml. of medium and one brain was added 10 μ l. of [¹⁴C]histidine (0.50 μ C). The system was incubated at 37° for 1, 1.5 or 2 hr. In some experiments the frog brain was homogenized in the tube and then incubated at 37° for various times.

After incubation, the system was made 80% with respect to ethanol and the brain homogenized. Four brains were incubated individually, homogenized and then pooled. The homogenates were subjected to the processes of extraction, column chromatography and concentration described by Abraham *et al.* (1962). The control or zero-time studies were performed under the same conditions with brain added after the system contained 80% of ethanol.

In later experiments, homogenized frog brains and a supernatant fraction prepared by centrifuging the homogenate at 10000g for 10 min. served as the source of the enzyme system. A modified medium of Utter, Harland & Reiner (1945) was used, containing (final concentrations) 50 mM-NaHCO₃, 8.9 mM-MgCl₂, 31 mM-glucose, 44 mM-nicotinamide, 0.7 mM-ATP, 0.5 mM-NAD⁺, 0.08 mM-CoA and 10 mM-sodium-potassium phosphate buffer, pH 7.3. The brains were homogenized in 5 vol. of this medium. To 0.9 ml. of homogenate were added 0.5 μ C of [¹⁴C]histidine, 3 μ moles of γ -aminobutyric acid or β -alanine and 3 μ moles of ATP, all in a total of 1.30 ml. After the incubation period, 4.0 ml. of ethanol was added and the tubes were placed in a boiling-water bath for 5 min. In the zero-time controls, the homogenate was added after the ethanol. After centrifugation the supernatants were treated as described for the experiments *in vivo*.

Chromatography methods. Columns (1.0 cm. $\times$ 60 cm.) of Dowex-50W (X4) saturated with 2,6-lutidine were used for the separation of histidine, carnosine and homocarnosine in the studies *in vivo* and *in vitro*, as described by Abraham *et al.* (1962).

Extracts were placed on the columns and eluted with 0.1 M-2,6-lutidine for approx. 20 hr., yielding a total eluate of 500–600 ml. The eluate was collected in fractions (5 ml.). Each tube was tested for the presence of amino acids or dipeptides by subjecting a 0.5 ml. sample to the ninhydrin or the Fast Red Salt TRN colour reaction or both (see below). The fractions containing histidine, carnosine and homocarnosine were concentrated *in vacuo* in a rotary evaporator. The samples, each dissolved in 2.0 ml. of water, were used for subsequent quantitative determinations.

Values were corrected for recovery by adding a known amount of homocarnosine, histidine and carnosine to the homogenates and taking the samples through the procedure. Recoveries were found to be 78% for homocarnosine, 86% for histidine and 83% for carnosine.

Quantitative determinations. The imidazole compounds were determined in the concentrated samples by the Fast Red Salt TRN method of Abraham *et al.* (1962). Standards

and blanks were prepared with 0.1 M-2,6-lutidine that had been eluted from the column and concentrated as were the samples of histidine, carnosine and homocarnosine. Corrections for losses during separation were made as described above.

Radioautography methods. Further evidence for the identity of the materials eluted from the columns was obtained by t.l.c. (Eastman Chromagram Sheet no. 606, silica gel) with the solvent system chloroform-methanol-aq. 17% (w/v) NH₃ (2:2:1, by vol.). Each sheet was placed in a cassette with a 8 in. $\times$ 10 in. sheet of non-screen X-ray film for 10–14 days and the film then developed. The dipeptides on the t.l.c. sheets were located by spraying with ninhydrin and their positions compared with the radioactive areas. The radioactive spots were scraped off and counted by liquid-scintillation techniques to determine the amount of radioactivity in the sample.

Liquid-scintillation techniques. The radioactivity of the concentrated samples was measured in a Packard Tri-Carb liquid-scintillation spectrometer. Samples (0.1–0.5 ml.) of the concentrated fractions were added to counting vials containing 10 ml. of the scintillation mixture described by Bruno & Christian (1961). This solvent system was also used when areas from the t.l.c. sheets were removed and placed in counting vials. Corrections for efficiency in counting were made by adding internal standards. In some cases, an efficiency curve was determined for use with the external standard supplied with the spectrometer.

RESULTS

Determination of homocarnosine, carnosine and histidine in brain of animals. The concentrations of homocarnosine, carnosine and histidine in rat, mouse, guinea-pig and frog brain were determined and compared with values from the literature (Table 1). Results are corrected for column recovery. In most cases, the values obtained in the present study were in agreement with those reported by others.

Incorporation of [¹⁴C]histidine into brain dipeptides in vivo. After the intracerebral injection of [¹⁴C]histidine, the specific radioactivity of the histidine in brain decreased, whereas the specific radioactivities of the carnosine and homocarnosine increased with time (Table 2). This indicated the incorporation of histidine into carnosine and homocarnosine. Control animals, given [¹⁴C]histidine intraperitoneally or endocardially, showed no apparent increase in the specific radioactivity of brain homocarnosine or carnosine. T.l.c. of the fractions followed by radioautography showed that the radioactivity corresponded to the positions of homocarnosine and carnosine.

To establish further the synthesis *in vivo* of [¹⁴C]-homocarnosine and [¹⁴C]carnosine from [¹⁴C]-histidine, the homocarnosine and carnosine samples from the column were hydrolysed in 6 M-hydrochloric acid (Pisano *et al.* 1961). The hydrolysate was concentrated and again subjected to the t.l.c. procedure and radioautography. The hydrolysed

Table 1. Concentrations of homocarnosine, carnosine and histidine in brain of various laboratory animals

The methods of separation and quantitative determination are given in the Materials and Methods section. Numbers in parentheses indicate the numbers of assays. The results are given as mean values $\pm$ S.E.M. References: (a) Kanazawa & Sano (1967); (b) Levi, Kander & Lajtha (1967); (c) Abraham *et al.* (1962); (d) Anastasi & Erspamer (1964).

	Homocarnosine (μ moles/100g. of tissue)		Carnosine (μ moles/100g. of tissue)
	Present investigation	Literature values	
Rat (9)	4.7 $\pm$ 0.3	5.6 ^a	1.5 $\pm$ 0.2
Mouse (2)	8.5	24.2 ^b	4.7
Guinea pig (6)	10.5 $\pm$ 0.8	21.1 ^b 5.9 ^a 18.3 ^c	1.7 $\pm$ 0.4
Frog (5)	190 $\pm$ 30	100.0 ^d 284.0 ^b	151 $\pm$ 11

	Histidine (μ moles/100g. of tissue)	
	Present investigation	Literature values
Rat (9)	3.7 $\pm$ 0.2	4.6 ^b
Mouse (5)	11.5 $\pm$ 0.7	8.1 ^b
Guinea pig (6)	6.7 $\pm$ 1.6	7.7 ^b
Frog (5)	30 $\pm$ 1.1	16.5 ^a

samples exhibited radioactivity only in the position of histidine.

Incorporation of [¹⁴C]histidine into brain dipeptides in vitro. After incubation *in vitro*, both intact and homogenized frog brain formed [¹⁴C]homocarnosine and [¹⁴C]carnosine from [¹⁴C]histidine (Table 3). Only a small quantity of radioactivity was found in zero-time controls in Expt. 1, and somewhat more in Expt. 2 and Expt. 3. Radioautography showed that this could be accounted for by contamination by [¹⁴C]histidine.

DISCUSSION

The results of these experiments indicate that frog brain can synthesize both carnosine and homocarnosine. There was some apparent variation in the activities of the brain supernatant preparations from different groups of frogs. A variation in enzyme activities from different groups of animals was observed by Kalyankar & Meister (1959) in the chick muscle system. The experiments reported here were done over a period of several months, and seasonal variation in frog brain

Table 2. Incorporation of [¹⁴C]histidine into brain dipeptides *in vivo*

L-[¹⁴C]Histidine (0.5 μ C) was injected into frogs as indicated. At the specified times the animals were killed and the brains removed and extracted by the procedures described in the Materials and Methods section.

Time after injection (hr.)	Route	Specific radioactivity (μ C/m-mole)		
		Histidine	Homocarnosine	Carnosine
0.5	Intracerebral	1446	2.6	3.1
	Intraperitoneal	0.6	0.01	0.002
1.0	Intracerebral	1173	4.4	6.1
	Intraperitoneal	2.2	0.06	0.02
	Intraperitoneal, three times	11.5	0.04	
	Into heart	0.5	0.02	0.008
2.0	Intracerebral	700	9.0	9.4
	Intraperitoneal	4.3	0.005	0.005

Table 3. Incorporation of [¹⁴C]histidine into dipeptides by frog brain tissue *in vitro*

The reactions were incubated at 37° for 1 hr., and the reaction was stopped by the addition of ethanol. The procedures are described in the text. (a) Incubated in medium of Krebs (1950) with 0.5 μ C of L-[¹⁴C]histidine (35 μ C/ μ mole); four incubated mixtures were pooled after the addition of ethanol to a final concentration of 80%. (b) Incubated in medium of Utter *et al.* (1945) with the addition of 3 μ moles of ATP and 1.0 μ C of L-[¹⁴C]histidine (10 μ C/ μ mole). —, Not measured.

Preparation	Radioactivity incorporated (d.p.m./g. of brain/hr.)	
	Homocarnosine	Carnosine
Expt. 1		
Whole brain control (a)	105	17
Whole brain (a)	1555	2474
Whole brain (a) (homogenized in reaction flask)	989	1023
Supernatant preparation from brain (b)	42094	71862
Expt. 2		
Supernatant preparation from brain (b), control	667	889
Supernatant preparation from brain (b)	2852	20083
Expt. 3		
Supernatant preparation from brain (b), control	392	—
Supernatant preparation from brain (b)	20235	39274

enzyme activity may have influenced the results. It is noteworthy that the ratio of carnosine to homocarnosine synthesized in 1 hr. *in vitro* is about the same as the ratio reported by Kalyankar & Meister (1959) for synthesis in 1 hr. catalysed by a more purified system from chick muscle.

Abraham *et al.* (1961) suggested that it is the availability of substrate that determines which dipeptides are synthesized in the various tissues. Since brain has a much higher concentration of γ -aminobutyric acid than of β -alanine, the predominant peptide is homocarnosine. However, a purified system from brain will have to be studied to establish whether the brain and muscle systems synthesizing histidine dipeptides are identical.

Some differences in the metabolism of homocarnosine and carnosine have been noted. Abraham, Pisano & Udenfriend (1964) found that carnosine, but not homocarnosine, is concentrated by rat brain slices. Marshall & Yockey (1968) showed that certain drugs significantly lower the concentration of rat brain homocarnosine without affecting those of carnosine. Nakajima, Wolfgram & Clark (1967), found that in bovine brain the molar ratio of homocarnosine to homoanserine is 4:1. In bovine skeletal muscle the ratio of carnosine to anserine is 6:1 (Crokaert, 1953). The function of these histidine dipeptides in excitable tissues remains an enigma; further study of their metabolism may help to elucidate their function.

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