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STUDIES OF METABOLISM OF IMIDAZOLE COMPOUNDS IN MOUSE BRAIN*

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Abstract—The distribution of imidazole derivatives was followed by thin-layer chromatography in suitably prepared extracts of brains of mice at various times after the parenteral administration of histidine, homocarnosine, carnosine, *N*-acetylhistamine and *N*-acetylhistidine. Under the conditions employed, evidence was obtained for the entry from blood of all of the above substances when 10 m-moles/kg was given intraperitoneally. The highest levels in whole brain of the substance administered were found for histidine, intermediate levels for homocarnosine and carnosine, and the lowest levels were found for *N*-acetylhistidine and *N*-acetylhistamine. In no instance were elevations of any imidazole substances other than those administered found in the brain extracts by the methods employed. Both mouse brain and kidney possessed much greater carnosinase than homocarnosinase activity. The rate of hydrolysis of glycylhistidine by brain was approximately nine times greater than that of carnosine, while homocarnosine, anserine and homoanserine were hydrolyzed, at most, to a slight extent by the preparations employed. The above are part of a series of experiments designed eventually to determine whether or not extracerebrally formed derivatives of histamine or histidine can enter the brain and be metabolized therein to histamine.

In previous studies we observed that [^{14}C]-labeled histidine intracerebrally administered to mice and frogs left the brain rapidly and, within the limitations of the methods employed, did not give rise to formation of any detectable amounts of histamine [1]. The latter finding was particularly perplexing to us because there is much evidence that suggests that histidine could be the immediate precursor of histamine in the brain [2–6]. A careful perusal of the literature convinced us that none of the published studies until this time have rigorously excluded the possibility of the entry of [^{14}C]histidine into the general circulation and the extracerebral formation of derivatives of [^{14}C]histamine or [^{14}C]histidine that can enter the brain and be metabolized therein to histamine. Blood-borne histamine probably is not the source of brain histamine, since histamine does not appear to cross the blood–brain barrier [2]. As a first step in exploring the above possibility, we have directed our attention to following imidazole compounds in the brain after the parenteral administration of histidine, homocarnosine, carnosine, *N*-acetylhistamine and *N*-acetylhistidine.

MATERIALS AND METHODS

Equipment, thin-layer chromatographic (t.l.c.) plates, column chromatography and visualization reagents. The following items were used: multiple rectangular tanks, E. Merck AG, Avicel 5537 and Silica gel 254 t.l.c. plates and Shandon-type spray gun (Brinkmann Instruments, Inc., Burlingame, Calif. 94010), Drummond disposable micropipettes, 1 and 2 μl (Van Waters & Rogers, Los Angeles, Calif. 90054), and Dowex AG-50 × 4 H^+, 200–400 mesh, and 0·7 × 10 cm glass columns (Bio-Rad, Richmond, Calif. 94804). Quantitative column chromatography of amino acids was performed on a Beckman model 117 amino acid analyzer. The unsubstituted imidazole derivatives were visualized with diazotized sulfanilic acid (a 1:1 ice-cold mixture of 1% sulfanilic acid in 2 N HCl and 5% sodium nitrite) or a 0·6% solution of Fast Red B (Sigma No. F 1125) in water. Substituted imidazole derivatives and amino acids were visualized with an 0·2% ninhydrin solution in absolute ethanol [7].

Chemicals. Imidazole derivatives, amino acids and sulfanilic acid were obtained from Sigma Chemical Co. (St. Louis, Mo. 63178). *N*-acetylhistamine was obtained from Calbiochem (San Diego, Calif. 92112). Other reagents were purchased from J. T. Baker Chemical Co. (c/o Van Waters & Rogers, Los Angeles, Calif. 90054).

Preparation of standards and samples. A detailed description for preparation of standards and samples was published previously [7]. The only change made in the present studies was that the perchloric acid extract was neutralized with 10 N KOH instead of solid K_2CO_3. When peptidase activity was measured, ethanol was used as deproteinizing agent instead of perchloric acid in order to avoid even small artifactual breakdown of the substrate.

In the majority of cases it was possible to employ one-dimensional thin-layer chromatograms because the small number of detectable imidazole derivatives could be well separated in this fashion.

Procedure for detection and quantitation of imidazole derivatives. After administration of varying amounts of histidine, homocarnosine, carnosine, *N*-acetylhistidine or *N*-acetylhistamine to 6- to 12-month-old male Swiss mice (Horton Laboratories, Oakland, Calif.),

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the animals were sacrificed at different time intervals by cervical dislocation. The brains (whole brain plus brain stem) were removed quickly, drawn through a saline solution and blotted carefully with filter paper to remove excess liquid and blood. Subsequently, the brains (two for each time interval) were placed in a homogenizer containing 3·0 ml of ice-cold 6% perchloric acid. In some instances, liver samples also were removed for analysis. Tissues were homogenized in a tissue grinder using 1 ml of 6% perchloric acid for each 200 mg of fresh weight of tissue. After deproteinization and centrifugation [1], all the supernatants were neutralized to pH 5·0 with 10 N KOH and evaporated to dryness in a rotary evaporator. The residues were redissolved in water so that 1 μl of the solution contained extract equivalent to 1 mg of fresh weight of tissue. Initially, an aliquot of 50 μl of the extract was placed directly on a Silica gel plate for one-dimensional chromatography for qualitative screening. A portion of the extract also was passed through an 0·7 × 3 cm column of Dow AG-50 × 4 H^+ and sequentially eluted with 20 ml water, 20 ml of 0·1 M pyridine, 20 ml of 1·0 M pyridine and then with either 1·0 or 3·0 M NH_4OH. The 0·1 M pyridine fraction will elute most of the acidic amino acids, organic acids and a few neutral amino acids. The first 7–8 ml of the 1·0 M pyridine fraction contained β-alanine, γ-aminobutyric acid, other neutral amino acids, *N*-acetylhistidine, and imidazolepropionic and imidazoleacetic acids. Histidine appeared in the last 12 ml of the 1·0 M pyridine fraction. Homocarnosine, carnosine and *N*-acetylhistamine were eluted with the first 6 ml of 1 M NH_4OH, while histamine appeared after at least 7 ml of 1 M NH_4OH had been passed through the column. If separation of histamine is not required, 3 M NH_4OH can be used. The fractions were concentrated and the imidazole derivatives and β-alanine and γ-aminobutyric acid eluted from the column were determined quantitatively by diazotization [7] and reaction with ninhydrin [8], respectively. Thin layer chromatography was used throughout these experiments as a monitoring tool and for semi-quantitative estimations in preliminary studies as well as for quantitative determinations, as described previously [7].

Determinations of peptidase activity. The method is essentially that of Smith [9] except that the products were determined by direct quantitative estimation, as described above, instead of by titration. Brain or kidney homogenates (10%) were prepared in 0·2 M Tris HCl, pH 8·02, containing 5×10^{-4} M $MnCl_2$. To 1·0 ml of the 10% tissue homogenate were added 0·5 ml Tris HCl buffer (0·2 M, pH 8·0) and 1·0 ml of 0·125 M substrate. Incubation was performed at 37° and the reaction terminated by the addition of 6·0 ml of 100% ethanol. After centrifugation at 3000 rev/min for 10 min, the supernatant was dried in a rotary evaporator. The dry extract then was redissolved in water to a desired vol. and used for t.l.c. or column chromatography. For quantitative estimates of peptidase activity, a Beckman autoanalyzer was used in conjunction with t.l.c.

RESULTS

Parenteral administration of imidazole derivatives. In preliminary experiments, 4 m-moles/kg of homocarnosine, carnosine, histidine or *N*-acetylhistamine was injected into the tail veins of mice, and the animals were sacrificed at 15 and 30 min after injection. Notable increases were found in brain contents of *N*-acetylhistamine and homocarnosine, but only slight increases in histidine and carnosine were observed. Changes in imidazole derivatives other than those administered were not detected by our procedures.

More extensive experiments were then performed with intraperitoneal administration of the above four substances and *N*-acetylhistidine. In one experiment, mice received 10 m-moles/kg of one of the above substances, and in each instance two animals were sacrificed at 5, 15, 30 and 60 min, respectively, after the injection. In the case of some of the substances, mice also were sacrificed at other times. Each of the injected substances was detected in the brain extracts at the earliest period studied, 5 min (Table 1). However, the time course of change differed for each of the compounds. Carnosine and homocarnosine attained the highest brain levels at 5 min, and the values declined thereafter, homocarnosine remaining elevated above control values for as long as 120 min. The levels of histidine and *N*-acetylhistamine rose more slowly, possibly indicating a slower rate of absorption from the peritoneal cavity. The highest concentration of any of the substances tested was

Table 1. Levels of imidazole derivatives in mouse brain at various times after intraperitoneal administration of 10 m-moles/kg

Time after administration of compound (min)	Content of compound in brain* (nmoles/g fresh weight)				
	Histidine†	Carnosine†	Homocarnosine†	*N*-acetyl-histidine†	*N*-acetyl histamine†
0	0‡	60	100	0	0
5	160	370	350	70	100
15	280	190	230	60	150
30		190			170
60	560	100	230	0	200
120	480		140		
180			120		130

* Two brains pooled for each time interval.

† Determined by t.l.c. as described previously [7]. Recovery of imidazole derivatives from tissue is > 95 per cent by this method.

‡ Below level of detection of procedures employed.

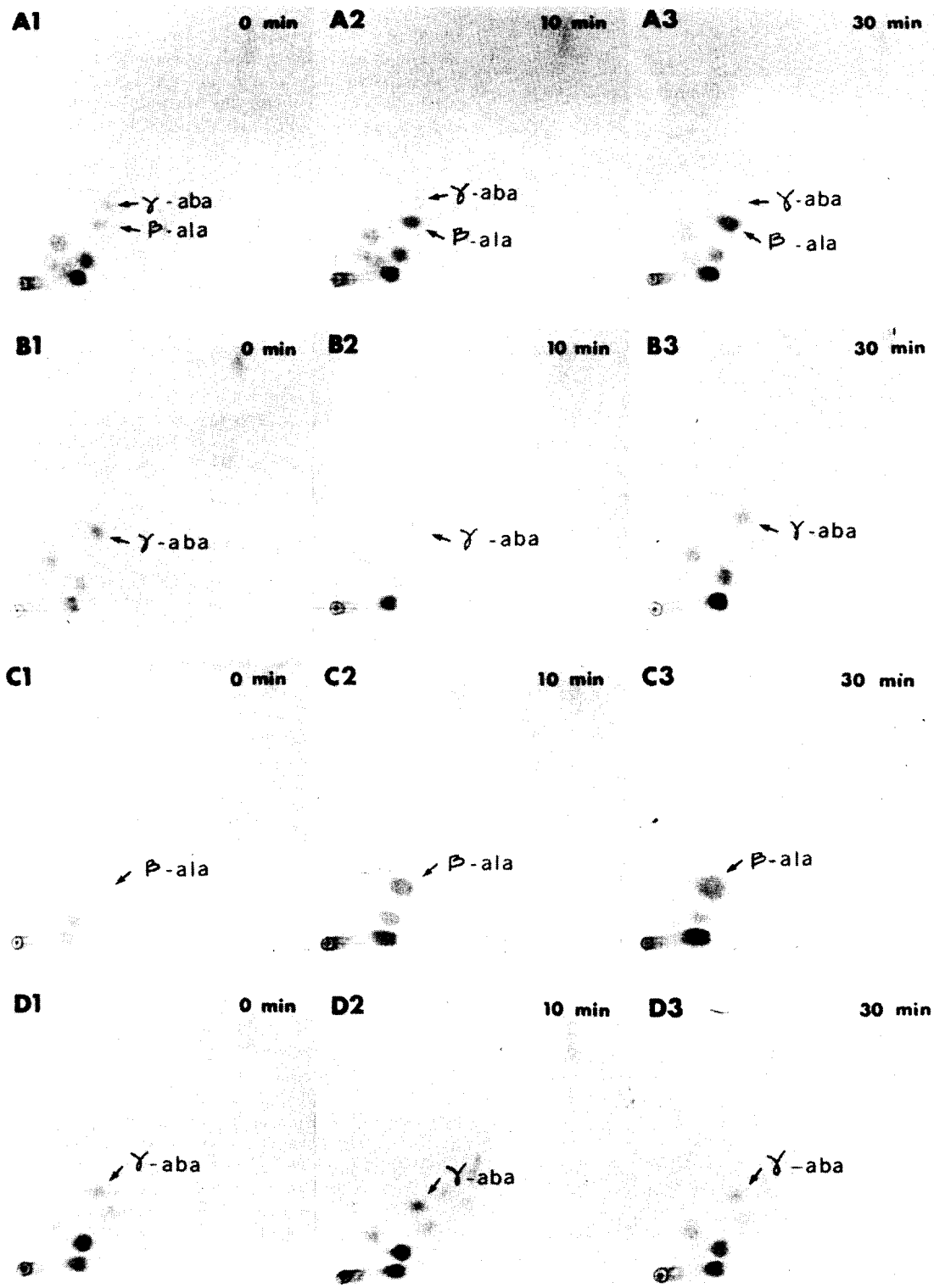

Fig. 1. Thin-layer chromatography of 0·1 M pyridine fractions of brain and kidney extracts on Avicel after incubation *in vitro* with carnosine and homocarnosine (see text). First dimension, 1 M NH_4OAc (pH 6·3)–isopropanol (30:70); second dimension, butanol–dioxane–formic acid–H_2O (40:40:5:15). Visualization reagent: ninhydrin (see text). (A) Carnosine—brain, (B) homocarnosine—brain, (C) carnosine—kidney, (D) homocarnosine—kidney.

Table 2. Histidine levels in mouse brain after intraperitoneal injection

Time after administration of histidine (min)	Content of histidine in brain* (nmoles/g fresh weight)		
	Dose administered (m-moles/kg)		
	2	4	10
30	100	390	1000
60	60	280	1170
120	0†	20	80

* Two brains pooled for the time interval.
† Below level of detection of procedures employed.

attained in the case of histidine. Elevations in histidine and *N*-acetylhistamine persisted for relatively long periods, little change occuring in content of the latter between 30 and 180 min. In contrast, *N*-acetylhistidine penetrated the brain to the least extent and was undetectable within 60 min after administration.

Livers of animals injected intraperitoneally with homocarnosine and *N*-acetylhistamine (10 m-moles/kg) showed a rapid increase followed by a rapid decline of the injected compounds. Since our principal interest was the metabolism of imidazole derivatives in brain tissue and the liver samples did not show changes in imidazole derivatives, the matter was not pursued further.

Since in previous experiments it was shown that there was poor retention of traces of labeled intracerebrally administered histidine [1], another experiment was performed in which 2, 4 and 10 m-moles/kg doses were adminstered intraperitoneally to mice and two mice from each group were sacrificed at 30, 60 and 120 min after injection (Table 2). At 30 min, the levels of brain histidine at the 4 and 10 m-moles/kg doses were almost exactly proportional to the dose injected. The rate of fall between 60 and 120 min was much smaller in the animals receiving 10 m-moles/kg than in the other two groups. This may be related to the blood levels. At the 2 m-moles/kg dose, the values at 60 min were 60 per cent of those at 30 min and fell to undetectable levels at 120 min. Those in mice receiving 4 m-moles/kg were 72 per cent of the 30-min level at 60 min, and only approximately 5 per cent at 120 min. On the other hand, the values in animals receiving 10 m-moles/kg actually rose between 30 and 60 min and fell to only approximately 65 per cent of the 30-min value at 120 min. Further studies will be aimed at relating blood levels of histidine attained to brain concentrations.

Carnosinase in mouse brain and kidney. There was a more rapid decline from the maximal levels of carnosine than of homocarnosine concentration in the brain between 5 and 60 min after intraperitoneal administration of the two dipeptides (Table 1). One of the possibilities suggested by the latter observation was that there might be a more rapid destruction of carnosine than of homocarnosine in the brain. In order to test the latter possibility, we measured carnosine and homocarnosine peptidase activities in mouse brain and kidney, essentially as described by Smith [9].

Both mouse brain and kidney homogenates possessed much greater carnosinase than homocarnosinase activity (Fig. 1). It is apparent from the t.l.c. plates that brain (A1-A3) and kidney (C1-C3) produced a large liberation of β-alanine from carnosine during the period of incubation, but that neither brain (B1-B3) nor kidney (D1-D3) caused any noticeable increase of γ-aminobutyric acid content over that of the control when homocarnosine was substrate. When carnosine was used as substrate, histidine formation also was demonstrated.

Preliminary attempts to concentrate the enzyme activity from kidney by $(NH_4)_2SO_4$fractionation have shown an increase of specific activity of carnosinase, but no corresponding enhancement of homocarnosinase. Separate experiments employing carnosine, homocarnosine, anserine, homoanserine or glycylhistidine with homogenates of mouse brain of kidney showed only carnosine and glycylhistidine to be hydrolyzed. The latter activities were observed in the presence of Mn^{2+} but not Zn^{2+}. The rate of hydrolysis of glycylhistidine by brain (1850 m-moles/g of fresh tissue/hr) was greater than that of carnosine (200 m-moles/g/hr), and the carnosine-splitting activity of kidney (690 m-moles/g/hr) was higher than that of brain.

DISCUSSION

In the present study, we have shown that, in addition to histidine, at least some bound forms of histidine and histamine (carnosine, homocarnosine, *N*-acetylhistidine and *N*-acetylhistamine) can cross the blood–brain barrier. Whether or not the above substances or related ones could serve as more immediate precursors of histamine in brain than free histidine, itself, can only be decided by experiments in which isotopic methods are employed to determine possible precursor–product relations. Measurements of increases in histamine content after administration of a substance could never be used as definite evidence for the precursor nature of the administered substance, since inhibition of metabolic transformation of rapidly turning over histamine also could lead to increases in histamine levels.

Because of lack of definitive evidence that histamine is made directly from histidine throughout the brain (see Ref. 10 for discussion), we consider it still a viable possibility that some bound form of histidine or histamine formed extracerebrally may be a direct precursor of brain histamine. Alternatively, it is possible that a bound form of histidine, such as carnosine, may be required to enter particular brain sites and to be hydrolyzed to give free histidine. Also, glycylhistidine can be hydrolyzed by brain preparations, although it has not yet been studied with regard to its ability to traverse the blood–brain barrier.

Our studies with mice indicate that brain peptidase is quite different from kidney peptidase [11, 12].

REFERENCES

1. J. N. A. van Balgooy, F. D. Marshall and E. Roberts, *J. Neurochem.* **19**, 2341 (1972).
2. R. W. Schayer and M. A. Reilly, *J. Neurochem.* **17**, 1649 (1970).
3. K. M. Taylor and S. H. Snyder, *J. Pharmac. exp. Ther.* **173**, 619 (1971).
4. C. K. Cohn, G. G. Ball and J. Hirsch, *Science, N.Y.* **180**, 179 (1973).

5. K. M. Taylor and S. H. Snyder, *J. Neurochem.* **19,** 341 (1972).
6. M. Baudry, M.-P. Martres and J.-C. Schwartz, *J. Neurochem.* **21,** 1301 (1973).
7. J. N. A. van Balgooy and E. Roberts, *Biochem. Pharmac.* **22,** 1405 (1973).
8. K. Esser, *J. Chromat.* **18,** 414 (1965).
9. E. L. Smith, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 93. Academic Press, New York (1955).
10. J. P. Green, in *Handbook of Neurochemistry* (Ed. A. Lajtha), Vol. 4, p. 224. Plenum Press, New York (1970).
11. H. T. Hanson and E. L. Smith, *J. biol. Chem.* **179,** 789 (1949).
12. P. J. van Heeswijk, J. M. F. Trijbels, E. D. A. M. Schretlen, P. J. J. van Munster and L. A. H. Monnens, *Acta. paediat. scand.* **58,** 584 (1969).