Neurochemical Research, Vol. 10, No. 3, 1985

Comment

MEASUREMENT OF BRAIN HISTAMINE: A Reappraisal

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Accepted October 18, 1984

INTRODUCTION

Problems of inconsistency exist among various laboratories (1–5) in the measurement of both brain and peripheral histamine (HA). For example, determinations of rat brain HA content between Hough and Domino (1) and Orr and Eichelman (2) are quite different, i.e., 42 vs 379 ng/g tissue. Both groups used a modification of the Taylor and Snyder (6) radioenzymatic assay for measuring HA. Another striking discrepancy among laboratories is the whole brain values reported for weanling guinea pigs. Blanco et al. (3) reported a value of 59 ng/g tissue for Tricolour guinea pigs, and Enwonwu and Okadigbo (4) obtained a value of 544 ng/g tissue for brain HA from Hartley guinea pigs. Both groups used a spectrofluorometric assay. It seems very unlikely that the difference in brain HA content between two strains of young guinea pigs is really an order of magnitude. Gleich and Hall (5) reported that different laboratories using similar and different methods for measuring plasma HA from identical samples obtained surprisingly different values. They concluded that reported absolute values for HA in biological specimens must be regarded with caution.

All of the pitfalls associated with the measurement of brain HA are not connected directly with the assay itself, but also with obtaining and pro-

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0364-3190/85/0300-0307$04.50/0

cessing the brain tissue. For instance, some investigators have studied the influence of animal-killing methods on results (1, 2, 7). Orr and Eichelman (2) asserted that high-volume homogenization of brain tissue resulted in more complete extraction of HA. The purposes of these comments are to describe a reliable procedure for the measurement of brain HA and to discuss factors that are important in realizing this goal.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats (200–400 g) from the Waisman Center Colony at the University of Wisconsin-Madison were killed by decapitation between 0900 and 1100 hours. The brains were immediately removed, dissected on ice, frozen on dry ice, placed in polypropylene tubes, and stored at −80°C until assayed. They were homogenized with either a Brinkmann polytron or a sonifier cell disruptor from Heat Systems Ultrasonics. Routinely, whole brains and cerebral cortices were homogenized in ice-cold deionized distilled water on ice with the polytron on setting "5" for 20 sec. Smaller regions were processed similarly except with the sonifer with a microtip at setting "3" for 20 sec. Homogenates in loosely covered tubes were placed in a boiling water bath for 10 min and centrifuged for 10 min at 40,000 *g* in a Sorvall RC-5B centrifuge. The supernatant fractions were decanted to polypropylene tubes and immediately assayed or stored at −20°C until assayed.

The assay procedure for brain HA that we have found to be sensitive and reliable is that essentially described previously by Orr and Pace (8). The following describes our modifications of that assay with additional comments. This brain HA assay is based on the enzymatic conversion of HA to [^{3}H]tele-methylhistamine (tMHA) using an ammonium sulfate fraction from rat kidney as our source of histamine-N-methyltransferase (HNMT) and S-adenosyl-L[*methyl*-^{3}H]methionine ([^{3}H]SAM).

The reaction was carried out in 400-μl polyethylene tubes in a volume of 40 μl. All additions to the assay, except for samples and blanks, were made with a 10-μl Hamilton repeating dispenser. The first addition was 10 μl of 0.3 M potassium phosphate buffer, pH 7.9, with or without added HA (100, 200, 400, and 800 pg as internal standards). Triplicate 20-μl samples of the tissue supernatant fraction were added to the tubes using a Gilson Microman (M-50). Blanks contained 20 μl of deionized distilled water instead of sample. Next, a reaction mixture containing HNMT, 0.5 μCi of 15 Ci/mmol [^{3}H]SAM (Amersham), and water to 10 μl was added. The amount of HNMT was that which yielded maximal conversion of HA to tMHA as determined experimentally. Prior to incubation, the tubes were vortexed while in the microfuge sliders and centrifuged briefly (< 5 sec) in a Beckman Microfuge B to remove any solution adhering to the side of the tubes and to assure adequate mixing. Following a 60-min incubation period at room temperature, 10 μl of 0.5 mg tMHA/ml of absolute ethanol were added to stop the reaction. After vortexing, tubes were placed on ice for 15 min and then centrifuged for 5 min at 4°C. Twenty μl from each supernatant were spotted on one of the channels of an LK5D TLC plate (Whatman Chemical Separation, Inc., Clifton, NJ) which had been carefully inspected before use for any gaps between the preadsorbent layer and the silica gel, activated at 120°C for 30 min, and cooled to room temperature. The spotted plates were air-dried in a fume hood for at least 45 min. In the summer when the ambient air was more humid, drying time was increased up to 90 min. If plates were not adequately dried, tMHA migrated unevenly and the band widths were wider and variable in width. The plates must *not* be dried in an oven at an elevated temperature. This destroyed the [^{3}H]SAM and gave very high blanks and uninterpretable results.

Plates were developed in TLC tanks at 4°C which helped reduce the blank. They were run to at least 12 cm in chloroform-methanol-concentrated ammonium hydroxide (12:7:1). The humidity affected the R_f value for tMHA and the width of the visualized bands. However, this did not affect the results.

After the plates were removed from the TLC tanks, they were sprayed with 0.2% ninhydrin in absolute ethanol and heated briefly at 120°C to visualize the tMHA spots. The tMHA spots were scraped from each channel into polypropylene minivials using an apparatus described by Hegstrand (9). The tMHA was eluted from the silica scrapings by adding 0.5 ml of absolute ethanol and shaking the minivials on a New Brunswick Scientific Shaker at 100 rpm for 10 min. Efficiency of tMHA elution from the scrapings was better with Whatman LK5D plates than with their harder LK6D plates or with Baker 7009-4 plates. Fluorescent indicators markedly reduced elution of tMHA. Following this step, 5 ml of Research Products International's 3a20 were added, and the radioactivity was determined using a scintillation counter.

Tissue HA levels were calculated from plots of dpm's versus internal HA standards. External standards were not appropriate because there was inhibition of HNMT in the presence of supernatants obtained from tissues. The amount of inhibition varied for different brain regions and peripheral tissues. The assay was sensitive to 50 pg of HA and linear to 800 pg of HA.

All values were determined by the TLC procedure described above except for some of those reported in Table III. The toluene:isoamyl alcohol procedure for measuring HA radioenzymatically was performed as reported by Orr and Eichelman (2).

RESULTS

We evaluated the effect of pH on the incorporation of tritiated methyl groups onto HA. Over the pH range tested (6.5 to 8.0), the pH curve was essentially flat. This indicated that when the measurement of histidine decarboxylase activity is coupled to this assay, the pH of the two assays can be identical (6).

Tissue preparation was an important step in the determination of brain HA. Homogenization of cerebral cortices using the polytron and sonifer were compared. The HA levels were the same with the two methods (27 $\pm$ 3 ng HA/g tissue for the polytron vs 26 $\pm$ 2 ng HA/g tissue for the sonifer).

Our results to determine whole brain HA in rats sacrificed by decapitation or microwave irradiation are presented in Table I. Comparison of our data with those from other laboratories showed a consistent marked increase in brain HA content from animals sacrificed by microwave irradiation.

Table II shows additional data on the influence of animal sacrifice methods on the determination of HA in the hypothalamus. The mean values for decapitation and for cervical dislocation followed by decapitation were very close to one another. However, there was considerable difference in the variability between these two methods. The SEM was 6.5% of the

TABLE I
WHOLE BRAIN HISTAMINE FROM RATS SACRIFICED BY DECAPITATION OR MICROWAVE IRRADIATION

Investigators	Decapitation (*n*) (ng/g tissue)	Microwave Irradiation (*n*) (ng/g tissue)	% Increase
Hegstrand and Hine	40 ± 4 (7)	226 ± 17 (7)[a]	465
Hough and Domino (1977)	42 ± 8[b]	124 ± 5 (8)	195
Orr and Eichelman (1979)	379 ± 53 (16)	767 ± 33 (4)	102
Oishi et al. (1983)	51 ± 3 (4–6)	336 ± 21 (4–6)	557

[a] $P < 0.001$; two-tailed Student's *t*-test between HA levels from rats sacrificed by decapitation and microwave irradiation.
[b] *n* not included in original paper.

mean for decapitation alone and 24% for cervical dislocation followed by decapitation. Animals sacrificed by CO_2 asphyxiation had hypothalmic HA values that were 40% greater than those of animals which were decapitated.

A comparison of two different HA assays and variable extraction volumes is presented in Table III. The toluene:isoamyl alcohol extraction method gave markedly higher levels of HA in whole brain and the values increased as volumes of water added to prepare the whole brain homogenate were increased. On the other hand, the HA procedure utilizing TLC to separate the [^{3}H]tMHA from other compounds produced consistent results, regardless of the homogenization volume used.

TABLE II
INFLUENCE OF SACRIFICE METHOD ON HYPOTHALAMIC HISTAMINE LEVELS

Method	*n*	Histamine (ng/g tissue)
Decapitation	4	261 ± 17
Cervical Dislocation Followed by Decapitation	4	252 ± 60
CO_2 Asphyxiation	4	357 ± 12[a]

[a] $P < 0.01$; two-tailed Student's *t*-test between CO_2 asphyxiation group and both other groups.

TABLE III

A COMPARISON OF THE HISTAMINE CONTENT FROM WHOLE BRAIN OBTAINED WITH DIFFERENT HOMOGENIZATION VOLUMES FOLLOWED BY TOLUENE-ISOAMYL ALCOHOL EXTRACTION OR THIN-LAYER CHROMATOGRAPHY

Homogenate Volume (ml)[a]	Toluene:Isoamyl Alcohol Extraction (ng/g tissue)	Thin-Layer Chromatography (ng/g tissue)
5	140 ± 21	44 ± 3
10	303 ± 44	47 ± 3
20	409 ± 60	48 ± 2

[a] Tissues were homogenized in ice-cold distilled deionized water/g tissue. The number of brains analyzed at each volume for each technique ranged from 3 to 16. Data are presented as mean ± SEM.

DISCUSSION

Because brain HA turns over rapidly (10), it is important to evaluate the effects of different methods of sacrifice on brain HA levels. Sacrifice methods include decapitation, cervical dislocation followed by decapitation, CO_2 asphyxiation, microwave irradiation, and liquid nitrogen immersion. Cervical dislocation prior to decapitation noticeably increased the variability in results. Microwave irradiation (Table I) and liquid nitrogen immersion (7) markedly increased whole brain HA levels. Oishi et al. (7) attributed this to the release of HA from mast cells in the dura that penetrate the brain; thus, the HA is included in whole brain homogenates. There is no apparent explanation for the different HA levels obtained after decapitation and CO_2 asphyxiation. This illustrates the importance of maintaining consistency in the method of animal sacrifice to make meaningful comparisons of data on brain HA levels.

We homogenized our brain tissues in water as did Orr and Pace (8) rather than in dilute phosphate buffer containing 0.1% Triton X-100 as did others using a radioenzymatic assay to measure HA (1, 6), or in perchloric acid as did investigators measuring HA fluorometrically (7). Taylor and Snyder (6) reported that Triton X-100 increases the HA extracted from brain and increases HNMT activity. We found no differences in the levels of HA extracted with water or with 0.1% Triton X-100 in dilute phosphate buffer. However, the methylation of HA in the presence of Triton X-100 was decreased about 20%, indicating HNMT was partially inhibited by Triton X-100. When we extracted brain tissue with perchloric acid, neutralized the extract with KOH, and assayed for HA radioenzymatically, we found almost no methylation of HA.

Our method of determining brain HA accurately measures the HA extracted by water. Very consistent values were obtained with this procedure. On the other hand, when exogenous HA was included in the water during homogenization of brain tissue, the amount of exogenous HA that was recovered was variable. Since our brain HA values were similar to a number of those reported (6, 10, 11), and since our consistency was excellent, we have chosen to report relative HA levels. Further studies on optimizing the extraction and recovery of HA might result in values closer to the absolute amount of HA present in brain tissues. However, for most experimental protocols, an accurate determination of relative values is what is most important. Our procedure insures that only endogenous HA rather than additional other compounds are measured.

We were able to reproduce the results of Orr and Eichelman (2) using their radioenzymatic assay (Table III). Although Orr and Eichelman used TLC to demonstrate that the toluene:isoamyl alcohol extract contained [^{3}H]tMHA, they did not quantitate their recovery from the TLC nor did they compare supernatants from various volumes of homogenization on TLC. We did the latter (Table III). Despite the fact that Orr and Eichelman had what seemed to be adequate controls and used internal standards, toluene:isoamyl alcohol apparently extracted one or more tritiated compounds, in addition to [^{3}H]tMHA when brain supernatants were analyzed for HA content. This did not happen with a chloroform extraction (6). However, chloroform extracts could include other tritiated compounds as well.

Verburg et al. (12) recently reported on optimizing the specificity for the radioenzymatic assay of HA. They also used a toluene-isoamyl alcohol extract but employed a 3:1 ratio instead of 1:1 ratio, as well as a highly purified preparation of HNMT. Whether these conditions would avoid the problems of Orr and Eichelman's method (2) remains to be determined. In addition, Verburg et al. (12) pointed out that N-α-methylhistamine (N-αMHA) is also a substrate for HNMT and therefore HA assays must distinguish between HA and N-αMHA. Since our TLC system readily separates HA from N-αMHA, we suspect that addition of another methyl group to N-αMHA would separate these two compounds as well. A dimethylated HA would be extracted by either chloroform or toluene-isoamyl alcohol, as is tMHA.

In addition, Verburg et al. (12) discussed the effect of temperature on the reaction of HNMT converting HA to tMHA. Lower temperatures favored HA methylation over N-αMHA methylation with 0°C being the optimum temperature. Since TLC separates those two products, that is not a concern for our assay. However, we ran the assay at room temperature which raised the sample to blank ratio, as compared to 37°C. At

0°C, the incorporation of tritiated methyl groups did not significantly increase the sample to blank ratio, but did necessitate longer incubation times to get comparable methylation.

We conclude that it is necessary to use some separation method when employing the radioenzymatic assay for determination of brain HA levels to be certain that only endogenous HA rather than N-αMHA, polyamines, or unknown compounds contribute to this measurement. TLC is a convenient and reliable way to achieve this important separation. In addition, animals should be sacrificed by decapitation, tissues should be frozen immediately, and supernatants should be prepared by homogenization in water with a polytron or sonifer followed by boiling and centrifugation. Such methods will insure consistent results that reflect relative endogenous brain HA.

ACKNOWLEDGMENTS

This paper was supported by the Veterans Administration and NIH grants MH 36787 and HD 03352. We wish to thank Tom Kalinke for his excellent technical assistance, and Cynthia Birch for editorial and secretarial assistance.

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