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SYNTHESIS OF TETRAHYDRO-β-CARBOLINES FROM INDOLEAMINES VIA ENZYMATIC FORMATION OF FORMALDEHYDE FROM 5-METHYLTETRAHYDROFOLIC ACID*

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Abstract—The products obtained on incubation of rat brain and rabbit lung with indoleamines and 5-methyltetrahydrofolic acid (MTHF) were found not to be the methylated compounds, as previously reported by others. Thin-layer chromatography of the basic extracts, as well as their acetylated derivatives, in solvent systems which adequately resolved very similar compounds, was employed to tentatively identify the products as tetrahydro-β-carboline derivatives. It is concluded that these products are formed by the condensation of the various indoleamines with formaldehyde generated enzymatically from MTHF, and that MTHF does not appear to be a significant methyl donor for biogenic amines.

Multiple reports from several laboratories in the past two years have described the use of 5-methyltetrahydrofolic acid (MTHF) as a donor for the methylation *in vitro* of biogenic amines [1–8]. Previously, this cofactor was believed to be involved only in methylation of amino acids and related substances [9]. The methylating enzyme(s) utilizing MTHF has been reported to be widely distributed in different tissues and in several species [4, 10]. Most of the work, however, has centered on its activity in brain [1, 2, 5–8], mainly because of the nature of the methylated products reportedly formed. These include bufotenine (*N*,*N*-dimethyl-5-hydroxytryptamine), *N*,*N*-dimethyltryptamine (DMT) and 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeO-DMT), compounds which are either known [11, 12] or suspected psychotomimetics [13].

These findings have served to bolster the "endogenous psychotogen" hypothesis of mental disorders such as schizophrenia [14] and have stirred speculation regarding the possible implication of MTHF and its associated enzyme(s) in the etiology of this disease [6, 15, 16].

In reviewing the literature of MTHF-mediated methylation of biogenic amines, we noted certain discrepancies and inconsistencies in the results which are worthy of discussion. One of the most obvious disparities was the position of methylation of such amine substrates as 5-hydroxytryptamine (5-HT); whereas one laboratory reported that both *N*- and *O*-methylation was observed [4, 5], another reported that MTHF mediated only *N*-methylation [2, 3, 17]. With regard to the activity of control assays in which heated protein preparations were used, three different observations were made: no activity [4], slight activity [1] and marked activity [7]. Furthermore, the enzyme displayed rather unusual kinetic properties [3]. Finally, although identification of the radioactive methylated products was accomplished by thin-layer chromatography (TLC), in some cases the correspondence of radioactive products and authentic standards was poor (see Figs. 1 and 2, and Ref. 5). We have recently described the enzymatic generation of [^{14}C]formaldehyde from [^{14}C]*S*-adenosylmethionine ([^{14}C]SAM) in erythrocyte hemolyzates [18]. When indoleamine substrates were present in the incubation medium, they formed condensation products with [^{14}C]formaldehyde to produce compounds which were difficult to distinguish chromatographically from the expected *N*-methylated substances. Criteria were established to differentiate the two kinds of products, however [18].

In subsequent experiments with [^{14}C]MTHF as methyl donor in brain and other tissues, we noted that these condensation products rather than the methylated ones were being formed. Elsewhere we have described the formation of such a condensation product, 6,7-dihydroxytetrahydroisoquinoline, when dopamine was used as the substrate in the incubation of brain tissue with [^{14}C]MTHF [19]. In this paper, we will present evidence that MTHF is not a significant methyl donor for biogenic amines; instead, this cofactor is aparently transformed by an enzymatic process to formaldehyde [20], which then reacts with indoleamine substrates such as tryptamine (TA) and 5-HT to form condensation products which are generally difficult to distinguish from the expected methylated substances. Since the completion of this study, it has come to our attention that others [21, 22] have made similar findings.

METHODS

Materials. The following were purchased from commercial sources: tryptamine hydrochloride (CalBio-

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chem), 5-hydroxytryptamine oxalate (Sigma), *N*-methyltryptamine (NMT) and 5-hydroxy-*N*-methyltryptamine (NMS) (Aldrich), [^{14}C]MTHF (sp. act. 60 mCi/m-mole, Amersham-Searle) and [^{14}C]SAM (sp. act. 50–60 mCi/m-mole, New England Nuclear). The [^{14}C]MTHF was dissolved in 40 μl mercaptoethanol, brought up to 5·0 ml with 0·25 M potassium phosphate buffer, pH 6·5, divided into aliquots and stored at −20°.

The tetrahydro-β-carbolines (THBC) were synthesized by the general method of Ho and Walker [23]. Each compound showed only one spot in the following solvent systems: acetone conc. NH_3 (99:1); ethyl acetate diethylamine–methanol (90:10:10); *n*-butanol–acetic acid–water (12:3:5); and isopropanol–10% ammonia water (200:10:20). The mass spectrum of each compound exhibited a molecular ion and base peak, respectively, at the expected m/e as follows: tetrahydro-β-carboline (THBC), 172, 143; 2-methyl THBC, 186, 143; 6-hydroxy THBC, 188, 159; and 6-hydroxy-2-methyl THBC, 202, 159.

Enzyme preparation and incubation conditions. Rat brain enzyme was prepared and incubated by the method of Laduron *et al.* [3] (homogenization of tissue in distilled H_2O, 30–65% ammonium sulfate pellet as enzyme source, pH of incubation 6·5). The somewhat different procedures used by others (homogenization in dilute Na phosphate, pH 7·9 use of 100,000 *g* supernatant as enzyme source, incubation in 0·005 M Na phosphate, pH 7·9, for shorter time periods [4]; homogenization in various buffers or distilled water, use of supernatant or various ammonium sulfate fractions as enzyme source, incubation at pH 6·5 to 8·0 and addition of EDTA [7]) gave the same results. The rabbit lung *N*-methylating enzyme first described by Axelrod [24] was prepared as previously described [18] except that lung tissue was homogenized in 5 volumes of isotonic KCl. Protein was measured by the method of Lowry *et al.* [25].

The assay system contained an aliquot of rat brain or rabbit lung enzyme preparation, 5mM indoleamine substrate, 8·3 μM [^{14}C]MTHF or 8·4 to 10·0 μM [^{14}C]SAM and 0·25 M potassium phosphate buffer (pH 6·5 when [^{14}C]MTHF was used and pH 7·9 when [^{14}C]SAM was the methyl donor), in a total volume of 1·0 ml. Incubation was carried out for 0·25 to 2 hr at 37° and the reaction was terminated by the addition of 1·0 ml of 0·5 M borate buffer, pH 10. The radioactive products were extracted into 6 ml ethyl acetate; 5 ml of the latter was evaporated to dryness with a stream of nitrogen at room temperature. (Extraction with the solvents employed by others [4,7], namely toluene–isoamyl alcohol (97:3) for the tryptamines and isoamyl alcohol alone for the hydroxy derivatives, did not qualitatively affect the results.) Radioactivity in the dried extracts was determined by scintillation counting after addition of 2 ml methanol (MeOH) and 10 ml LSC Complete (Yorktown Research). Heated protein controls were run concurrently. Efficiency of the scintillation system for [^{14}C] was 88 per cent.

Chromatography. Thin-layer chromatography (TLC) of the basic extracts, after addition of standards, was performed on Silica gel Chromagram sheets (Eastman, with fluorescent indicator) in acetone–conc. ammonia (99:1); TLC of acetylated products was run on Merck Silica gel plates (with fluorescent indicator) in $CHCl_3$–diethylamine (90:10). Spots were visualized by u.v. detection. Acetylation of the basic extracts was conducted as previously described in our experiments with dopamine [19].

RESULTS

TLC of the basic extracts from various incubations. Of the many different solvent systems we tried, the best separation of the various possible products from each other and from the starting substrate was obtained in acetone–conc. ammonia (99:1) on Chromagram sheets. Figure 1 shows the results obtained with 5-HT as substrate. When brain enzyme was incubated with [^{14}C]MTHF, the product ran exactly like 6-hydroxy-1,2,3,4-tetrahydro-β-carboline (6-OH THBC) (Fig. 1a); incubation of 5-HT with rabbit lung enzyme and [^{14}C]SAM, however, resulted in the formation of the expected *N*-methylserotonin (NMS) (Fig. 1b). To show that formation of the β-carboline was due to the methyl donor and not to the different tissue we also incubated 5-HT with lung enzyme and [^{14}C]MTHF; in this case, the β-carboline was again formed (Fig. 1c). The minor peak (Fig. 1a and 1c) also did not correspond to the *O*-methylated β-carboline (i.e. 6-methoxy THBC), since the latter ran much more slowly (R_f 0·22).

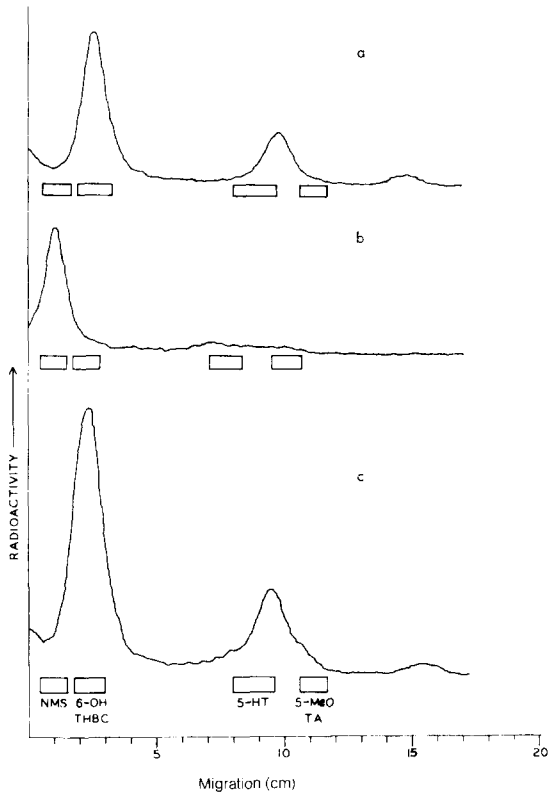

Fig. 1. Radiochromatogram scans of the product extracted from incubation of 5-HT with: (a) rat brain and [^{14}C]MTHF; (b) rabbit lung and [^{14}C]SAM; and (c) rabbit lung and [^{14}C]MTHF. TLC as described in Methods. Abbreviations: NMS, *N*-methylserotonin; 6-OH THBC, 6-hydroxy-1,2,3,4-tetrahydro-β-carboline; and 5-MeO TA, 5-methoxytryptamine.

Table 1. Radioactivity (cpm) in various product areas after thin-layer chromatography*

Enzyme source	Methyl donor	Substrate (R_f)	*N*-methyl product (R_f)	*O*-methyl product (R_f)	β-Carboline product (R_f)
Rat brain	[^{14}C]MTHF	TA (0·80)	1,379 (0·16)	–	8,461 (0·26)
		NMT (0·16)	404 (0·63)	—	5,999 (0·82)
		5-HT (0·52)	2,230 (0·07)	946 (0·65)	31,438 (0·14)
		NMS (0·07)	3,342 (0·35)	1,797 (0·13)	16,979 (0·49)
Rabbit lung	[^{14}C]SAM†	TA	25,202	—	461
		NMT	19,037	—	25
		5-HT	21,672	46	285
		NMS	25,468	268	120
Rabbit lung	[^{14}C]MTHF	TA	1,355	—	11,829
		NMT	501	–	10,676
		5-HT	3,523	987	52·148
		NMS	9,395	4,285	41,611

* Rat brain incubations contained 3·5 mg partially purified enzyme (see Methods). Rabbit lung incubations contained 0·5 ml (6·3 mg protein) of a 100,000 *g* supernatant. Other conditions are described in Methods.

† Total sample applied for MTHF experiments, but only 10% in SAM experiment.

Incubation of the other indoleamines gave similar results (Table 1). Usually in each case the major product was the β-carboline. Varying amounts of other unidentified products were also formed, but they never coincided with the expected *N*- or *O*-methylated products on TLC. Although with NMS as substrate we obtained the expected β-carboline, a second peak running slightly faster was also obtained, which often was larger than the β-carboline peak. This second peak was identical in all respects to the single peak obtained in our previous blood studies using NMS and [^{14}C]SAM [18], which was also shown to be a formaldehyde condensation adduct with NMS, but of unknown structure [18].

Some radioactivity was also found after elution of areas of the chromatograms corresponding to the *N*-methylated compounds (Table 1), although no peak was found in these areas on scanning. This probably represents trailing of the radioactivity from the β-carboline areas (compare R_f values of these products in Table 1).

TLC of the basic extracts after acetylation. When the extracts were acetylated (after addition of the appropriate non-radioactive β-carboline standard) and chromatographed, the major peak of radioactivity was observed to migrate exactly with the cold acetylated standard (Fig. 2). (In the case of NMT as substrate, of course, the product after acetylation was still the free tertiary amine which cannot be acetylated under the conditions employed.) The acetylated substrates are well separated from the product areas. Minor unidentified peaks are visible, but in the case of NMS the second peak is quite large (as discussed above for the free amines) and in some experiments was much larger than the β-carboline peak.

Inhibition of β-carboline formation by cysteine. Cysteine has been showed to react very rapidly with formaldehyde at neutral pH to form thiazolidine-4-carboxylic acid [26]. As such, it was used to inhibit the condensation of dopaldehyde with dopamine to form tetra-hydropapaveroline during assay of monoamine oxidase (MAO) activity in rat brain [27]. Addition of 5 mM cysteine to brain enzyme incubations of NMS and [^{14}C]MTHF resulted in a large decrease in extractable cpm (Table 2). This would be expected since the thiazolidine-4-carboxylic acid formed is an amino acid which would not extract into organic solvents. Addition of cysteine to rabbit lung incubations with [^{14}C]SAM, however, did not show this effect, while rabbit lung with [^{14}C]MTHF gave the same results as brain with [^{14}C]MTHF (Table 2). The results are consistent with the hypothesis that [^{14}C]MTHF is transformed to [^{14}C]formaldehyde [20].

DISCUSSION

The data amply demonstrate that MTHF is not a methyl donor for biogenic amines. Instead, formaldehyde is generated enzymatically [19, 20] which then reacts with the substrate indoleamines to form β-carboline derivatives, as well as other unknown condensation products [18]. Previous reports that MTHF is involved in *N*- [1–8] and *O*-methylation [4–6] of these compounds to form psychotomimetic substances are therefore, concluded to be based on the use of solvent systems for TLC which are inadequate to resolve the expected methylated products from the unexpected tetrahydro-β-carbolines. The observed poor overlap of some of the radioactive products with authentic standards [5] is thus understandable.

That formaldehyde is generated enzymatically from MTHF has been demonstrated by us previously [19]. With regard to boiled protein blanks, we noted above that different laboratories have reported differing amounts of activity in such samples. Indeed, we have found that such controls do yield β-carboline products (verified by TLC) but that this activity is highly variable from experiment to experiment [19]. The blank activity can be as low as 3 per cent and as high as 20 per cent of the sample activity. At this time we have no explanation for this observation.

The production of tetrahydro-β-carbolines or tetrahydroisoquinolines from indoleamine or catecholamine neurotransmitters and their derivatives may, nevertheless, be interesting in view of the reported pharmacological activity of such compounds [28–30].

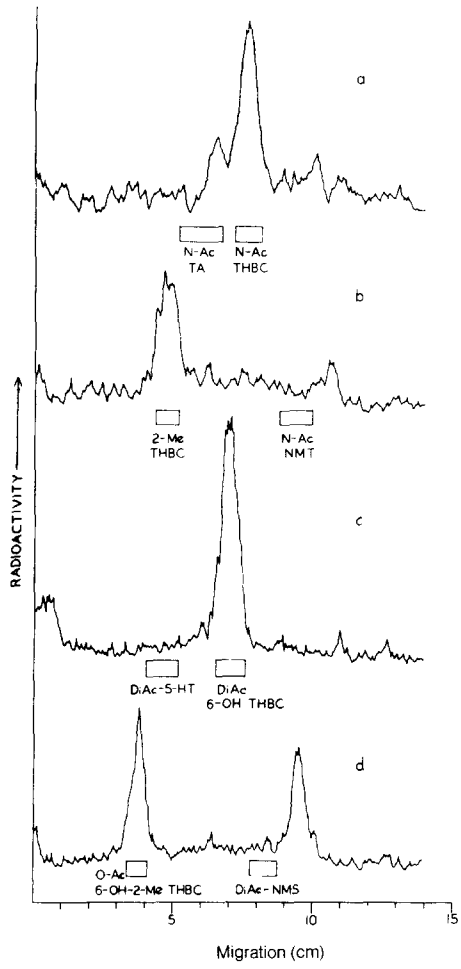

Fig. 2. Radiochromatograms of the acetylated extracts of incubations of rat brain with [^{14}C]MTHF and: (a) tryptamine; (b) *N*-methyltryptamine; (c) 5-HT; and (d) *N*-methylserotonin. TLC as described in Methods. Abbreviations: N-Ac TA, *N*-acetyltryptamine; N-Ac THBC, *N*-acetyltetrahydro-β-carboline; 2-Me THBC, 2-methyltetrahydro-β-carboline; N-Ac NMT, *N*-acetyl-*N*-methyltryptamine; DiAc-5-HT, *N,O*-diacetyl-5-hydroxytryptamine; DiAc 6-OH THBC, *N,O*-diacetyl-6-hydroxytetrahydro-β-carboline; O-Ac 6-OH-2-Me THBC, *O*-acetyl-6-hydroxy-2-methyltetrahydro-β-carboline; and DiAc-NMS, *N-O*-diacetyl-*N*-methylserotonin.

Table 2. Effect of cysteine on extraction of radioactivity from incubation mixtures*

Enzyme source	Methyl donor	Cysteine (5 mM)	Radioactivity extracted (cpm)
Rat brain	[^{14}C]MTHF	−	26,412
		+	3,675
Rabbit lung	[^{14}C]SAM	−	420,175
		+	450,975
Rabbit lung	[^{14}C]MTHF	−	79,110
		+	2,875

* NMS (5 mM) was used in these experiments. Other conditions are as described in Methods.

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