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OXIDATIVE METABOLISM OF MESCALINE IN THE CENTRAL NERVOUS SYSTEM—V

IN VITRO DEAMINATION OF MESCALINE TO 3,4,5-TRIMETHOXY-BENZOIC ACID

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Abstract—3,4,5-Trimethoxy-benzoic acid (3,4,5-TMBA) besides 3,4,5-trimethoxy-phenylacetic acid (3,4,5-TMPAA) is formed from mescaline during its incubation with preparations of different mouse tissues. Brain has the highest capacity to form 3,4,5-TMBA among the tissues so far studied. The enzyme system which catalyses the oxidative deamination of mescaline to 3,4,5-TMBA is localized in the nuclear and microsomal fractions of brain. Oxygen, NAD(P)H and unidentified factors of the high speed supernatant of brain extract are necessary for optimal reaction conditions. 3,4,5-TMBA formation was inhibited by SKF 525-A, but not by MAO and DAO inhibitors. The similarities and dissimilarities of the enzymic deamination of amphetamine and mescaline are discussed.

Mescaline metabolism in brain is a more complex process than is generally believed. Besides acetylation to *N*-acetylmescaline and oxidative deamination by MAO to 3,4,5-trimethoxy-phenylacetic acid (3,4,5-TMPAA), side chain degradation occurs *in vivo*, especially in brain, whereby 3,4,5-trimethoxy-benzoic acid (3,4,5-TMBA) is formed as the end-product of the reaction sequence [1].

From a comparison of the capacity of the cellular organelle fractions to oxidatively degrade mescaline, Zeller *et al.* [2] assumed the existence of an oxidative pathway parallel to the MAO and DAO catalysed deaminations; the authors suggested an eventual role of the amphetamine degrading microsomal enzyme in mescaline catabolism, which was characterized by Axelrod [3]. With the exception of our finding [1] there was, however, no experimental evidence which suggested the heterogeneity of the product of oxidative mescaline metabolism. The detection of 3,4,5-TMBA as a normal mescaline catabolite prompted us to characterize and localize the enzyme system which is responsible for the formation of this compound.

Abbreviations: t.l.c., thin-layer chromatography; MS, mass spectrometry; MAO, monoamine oxidase; DAO, diamine oxidase; 3,4,5-TMPAA, 3,4,5-trimethoxy-phenylacetic acid; 3,4,5-TMBA, 3,4,5-trimethoxy-benzoic acid; NAD, NADH, nicotinamide adenine dinucleotide and reduced form; NADP, NADPH, nicotinamide adenine dinucleotide phosphate and reduced form; DANS-Cl, 5-dimethylamino-naphthalene-1-sulphonyl-chloride.

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MATERIALS AND METHODS

Radiochemicals and reagents. [8-^{14}C]Mescaline.HCl (sp. act. 4·5 Ci/mole) was purchased from New England Nuclear Corporation, Boston. [2,6-^{3}H]Mescaline.HCl (sp. act. 36·5 Ci/mole) was prepared as described previously [4]. The specificity of the tritium labelling in the benzene nucleus was ensured and tritium exchange during the analytical procedure was excluded [5]. For incubation with tissue preparations a 1:1 mixture of [8-^{14}C]- and [2,6-^{3}H]mescaline.HCl was prepared, which was purified by t.l.c. (silica gel G, 1-mm thick layer; solvent; *t*-butanol–2-oxo-butane–25% ammonia–water (4:3:2:1). The ^{3}H/^{14}C ratio of the mixture was determined by liquid scintillation counting, using the two channel method. It was further checked by preparation of the DANS-derivative [6] of the radioactive mescaline, purification of the derivative by t.l.c., and liquid scintillation counting.

Tranylcypromine (*trans*-2-amino-1-phenylcyclopropane.HCl) and SKF 525-A (2-diethylaminoethyl-α,α-diphenyl valerate) was obtained from Smith, Kline & French Labs., Philadelphia, Disulfiram (*bis*-(diethylthiocarbamoyl)-disulfide); NADPH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (from yeast) were from Boehringer, Mannheim. All other chemicals were obtained from E. Merck, Darmstadt.

Tissue preparations. The organs of male albino mice (NMRI, Ges. f. Versuchstierzucht, Hannover) weighing 25–30 g were used. They were homogenized immediately after their excision either with 3 volumes of ice-cold phosphate buffer (66 mM; pH 7·2) or with 10

volumes of 0·25 M sucrose. Nuclear, mitochondrial and microsomal fractions were obtained by differential centrifugation of the homogenates in sucrose according to Schneider and Hogeboom [7]. The pellets of the different fractions were rehomogenized with sucrose and recentrifuged, and then suspended in 66 mM phosphate buffer.

Purified nuclei were obtained from the nuclear fraction of rat liver by centrifugation in sucrose and Ficoll density gradients [8].

High speed supernatants of brain and liver homogenates in phosphate buffer were prepared by centrifugation at 100,000 *g* for 60 min.

Incubation mixtures. 0·1-ml portions of suspensions of the tissue preparations in 66 mM phosphate buffer or high speed supernatant of brain and liver homogenate contained 1·25 mM $MgCl_2$, 1·25 mM nicotinamide, and if added, 0·25 mM NADP, NADPH or NADH [3]. Inhibitor concentration was 1 mM; mescaline.HCl concentration was 2 or 5 mM. The incubation mixtures were shaken in sealed reagent tubes in a water bath at 38 ± 0·5° for different time intervals. The enzyme reaction was stopped by addition of 0·2 ml 0·2 N $HClO_4$.

Protein determination. Protein was determined by a modified Lowry method [9].

Determination of 3,4,5-TMBA and 3,4,5-TMPAA in incubation mixtures. Anionic plus neutral reaction products of radioactive mescaline were separated from the cationic compounds by ion-exchange chromatography as described previously [10]. The columns (5 × 120 mm) were filled with a slurry of cellulose phosphate (Whatman P11, W. & R. Balston, Maidstone) in water–methanol (4:1). Elution of the anionic plus neutral fraction was achieved with 3 ml of the water–methanol mixture at a rate of 2 ml/cm²/hr. The eluate was mixed with 1 ml of a 10 per cent solution in chloroform of Amberlite LA-2 (Serva, Heidelberg) liquid anion exchange resin. The water phase was discarded; the chloroform phase was washed with 4 ml of water. After evaporation of the solvent in a stream of air, 10 ml of Aquasol I (New England Nuclear Corp., Boston) were added and the $^3H/^{14}C$ ratio was determined by liquid scintillation counting, using the two channel method. Counting efficiency was determined by addition of [^{3}H] and [^{14}C]toluene as internal standards. Recovery of [^{3}H]3,4,5-TMPAA was >90 per cent.

In the reaction mixtures, in which the oxidation reaction was stopped immediately after the addition of the radioactive substrate, 0·06 per cent of the total radioactivity was found in the anion fraction. This amount of radioactivity (zero value) was subtracted from the radioactivity measured in the anion fraction of each incubated reaction mixture.

Identification of 3,4,5-TMBA. Twenty-five incubated reaction mixtures with suspensions of brain nuclei (from 4·2 g of mouse brain; composition of the reaction mixtures and incubation conditions were the same as those indicated in Table 2) were centrifuged after acidification with 0·2 N $HClO_4$. The supernatants were combined, and, after saturation with $Na_2SO_4.10H_2O$, extracted with two 50-ml portions of ethyl acetate. The residue of the ethyl acetate extracts was dissolved in 20 ml of ethanol. This solution was evaporated to a volume of 0·5 ml, after its clarification by centrifugation, and then applied in a 10-cm-long streak to a thin-layer plate (1 mm thick silica gel G_{F254}). Small spots of 3,4,5-TMBA and 3,4,5-TMPAA were also applied near the plate edge as reference compounds. The plate was placed into a Br_2 vapour saturated chromatographic tank for 5 min in order to transform 3,4,5-TMBA and 3,4,5-TMPAA into their bromination products [1]. After chromatographic separation of the bromination products with *t*-butanol–2-oxo-butane–25% ammonia–water (4:3:2:1) and localization of the 1,2,3-trimethoxy-4,5,6-tribromobenzene zone by means of fluorescence quenching, the reaction product of 3,4,5-TMBA was eluted from the silica gel with ethyl acetate. This extract was subjected to bi-dimensional t.l.c. using acetone–methanol (19:1) in the first dimension and benzene–methanol (5:1) in the second dimension as solvents. The spot corresponding in its chromatographic mobility to 1,2,3-trimethoxy-4,5,6-tribromobenzene was again eluted with ethyl acetate. A mass spectrum was prepared from the residue of this extract.

Mass spectrometry. Mass spectra were prepared with the CH5 mass spectrometer (Varian, MAT, Bremen) at an electron beam energy of 70 eV. The temperature of the electron source was 250°.

RESULTS

Bromination of 3,4,5-TMBA to 1,2,3-trimethoxy-4,5,6-tribromobenzene and mass spectrometry of this derivative, show that 3,4,5-TMBA is not only formed *in vivo* from mescaline [1], but is also formed *in vitro* under the reaction conditions indicated in Table 2. (The mass spectrum of the compound isolated from 25 standard incubation mixtures was identical with that of an authentic sample of 1,2,3-trimethoxy-4,5,6-tribromobenzene (see Fig. 4 of Ref. 1); the *m/e* value of the peaks at 344 and 346 has to be corrected, however to 346 and 348, since an error was made during the drawing of this figure.)

The quantitative determination of 3,4,5-TMBA in the reaction mixture is based on the estimation of the loss of radioactive carbon of [8. ^{14}C] mescaline. A typical example is the following: after the incubation of 0·1 ml of brain homogenate, with 5 mM [^{3}H, ^{14}C] mescaline.HCl corresponding to 9·2 μCi ^{3}H and 1·12 μCi ^{14}C, the anion fraction contained 99,800 dis/min ^{3}H and 10,200 dis/min ^{14}C. Since the $^3H/^{14}C$-ratio of the substrate was 8·2 and that of the anion fraction 9·8, a loss of 1900 dis/min ^{14}C is calculated. This amount of radioactivity is equivalent to 0·21 nmole 3,4,5-TMBA. The loss of ^{14}C in the anion fraction can be equated to the formation of 3,4,5-TMBA under the following premises: (a) The anion fraction contains, in accordance with our previous *in vivo* study [1], only

Table 1. Formation of 3,4,5-trimethoxy-benzoic acid (3,4,5-TMBA) and 3,4,5-trimethoxyphenylacetic acid (3,4,5-TMPAA) from mescaline (3,4,5-trimethoxy-β-phenylethylamine) by tissue homogenates

Tissue	Rate of formation (nmole/18 mg fresh wt/3 hr)		Per cent of anionic metabolites	
	3,4,5-TMBA	3,4,5-TMPAA	3,4,5-TMBA	3,4,5-TMPAA
Brain	0·40 ± 0·01	2·65 ± 0·2	13	87
Liver	0·30 ± 0·02	4·42 ± 0·4	6	94
Kidney	0·12 ± 0·05	1·11 ± 0·1	9	91
Heart	0·07 ± 0·02	1·40 ± 0·2	5	95

Incubation mixture: 0·1 ml (5 mM[2,6-^{3}H]/[8-^{14}H]mescaline.HCl (9·2 μCi ^{3}H, 1·12 μCi ^{14}C); 18 mg tissue homogenized in 66 mM phosphate buffer pH 7·2). Incubation conditions: shaking at 38 ± 0·5° for 3 hr.

The values are means ± S.D. of three experiments.

Table 2. Distribution of mescaline oxidizing activity in organelle fractions of mouse brain. (Formation of 3,4,5-TMBA and 3,4,5-TMPAA)

Fraction	3,4,5-TMBA (nmole/mg protein/2 hr)	3,4,5-TMPAA (nmole/mg protein/2 hr)
Homogenate	0·14 ± 0·02	0·87 ± 0·04
Nuclei in phosphate buffer	0·22 ± 0·05	1·36 ± 0·2
in brain extract*	0·42 ± 0·1	2·47 ± 0·3
Mitochondria in phosphate buffer	0·002 ± 0·001	1·29 ± 0·1
in brain extract*	0·003 ± 0·001	1·38 ± 0·02
Microsomes in phosphate buffer	0·012 ± 0·002	0·71 ± 0·02
in brain extract*	0·054 ± 0·01	0·51 ± 0·03

Incubation mixture: 0·1 ml (2 mM[2,6-^{3}H]/[8-^{14}C]mescaline.HCl (3·7 μCi ^{3}H, 0·45 μCi ^{14}C); 1·25 mM $MgCl_2$; 1·25 mM nicotinamide, 0·25 mM NADP. The tissue preparations were suspended either in 66 mM phosphate buffer pH 7·2 or the high speed supernatant of brain homogenate. Incubation conditions: shaking at 38° for 2 hr.

The values are means ± S.D. of three determinations.

* No significant influence of rat liver extract on 3,4,5-TMBA formation.

Table 3. The influence of NADH and NADPH on the formation of 3,4,5-TMBA and 3,4,5-TMPAA from mescaline by brain microsomes and purified liver cell nuclei

	Brain microsomes		Liver cell nuclei	
	3,4,5-TMBA (nmole/mg protein/2 hr)	3,4,5-TMPAA	3,4,5-TMBA (nmole/mg protein/2 hr)	3,4,5-TMPAA
NADP (0·25 mM), phosphate buffer	0·012 ± 0·004	0·71 ± 0·02	0·086 ± 0·02	0·85 ± 0·05
NADP (0·25 mM), brain extract	0·054 ± 0·01	0·51 ± 0·03	0·14 ± 0·01	1·1 ± 0·03
NADPH (0·25 mM), phosphate buffer	0·024 ± 0·008	0·67 ± 0·01	0·13 ± 0·01	0·95 ± 0·01
NADP (0·25 mM), glucose-6-phosphate (1·25 mM), phosphate buffer	0·009 ± 0·005	0·69 ± 0·09		
NADP (0·25 mM), glucose-6-phosphate (1·25 mM), glucose-6-phosphate dehydrogenase (0·1 mg), phosphate buffer	0·028 ± 0·006	0·72 ± 0·1		
NADH (0·25 mM), phosphate buffer	0·028 ± 0·01	0·53 ± 0·03		

Incubation mixture: 0·1 ml (2 mM[2,6-^{3}H]/[8-^{14}H]mescaline.HCl (3·7 μCi ^{3}H, 0·45 μCi ^{14}C), 1·25 mM $MgCl_2$, 1·25 mM nicotinamide. The tissue preparations were suspended either in 66 mM phosphate buffer pH 7·2 or in the high speed supernatant of brain homogenate. Incubation conditions: shaking at 38° for 2 hr.

Values are means ± S.D. of three determinations.

3,4,5-TMBA and 3,4,5-TMPAA, and eventually *O*-demethylated acids. *O*-Demethylated mescaline in the anion fraction would feign a diminished extent of side chain degradation. (b) The C_1-fragment of mescaline degradation does not appear in the anion fraction. Its whereabouts are at present unknown. $^{14}CO_2$ is not formed in the *in vitro* experiments, in contrast to the *in vivo* experiments, where we were able to show the formation of $^{14}CO_2$ from the C_8-carbon atom of mescaline [1].

Homogenates of different tissues differ in their capacity to form 3,4,5-TMBA and 3,4,5-TMPAA. As can be seen in Table 1, brain homogenates show the highest rate and the highest proportion of 3,4,5-TMBA-formation.

Table 2 shows the distribution of the 3,4,5-TMBA and 3,4,5-TMPAA forming capacity among the subcellular fractions of brain homogenates. Nuclear and microsomal fractions were most active in forming 3,4,5-TMBA, whereas mitochondria and cytosol were inactive. The mescaline degrading capacity of the nuclear fraction is not related to impurities (erythrocytes, coarse cell fragments etc.). Highly purified nuclei [8] also showed 3,4,5-TMBA formation (Table 3). Substitution of phosphate buffer by brain extract had a considerable activating effect on the oxidative degradation of mescaline by subcellular components (Table 2). 3,4,5-TMBA formation was activated in all fractions, whereas 3,4,5-TMPAA formation was only activated in the nuclear fraction.

Thoroughly washed microsomes lost their 3,4,5-TMBA forming capacity, and no significant formation of 3,4,5-TMBA was observed under anaerobic conditions. Addition of $MgCl_2$, nicotinamide and NADPH and aeration completely restored the formation of 3,4,5-TMBA. NADPH could be substituted by an NADPH generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) as can be seen in Table 3. Substitution of phosphate buffer by brain extract had an additional activating effect on the microsomal system, whereas this effect was not observed with purified nuclei (Table 3).

Typical MAO or DAO inhibitors did not influence the formation of 3,4,5-TMBA from mescaline, whereas at the same time 3,4,5-TMPAA formation was nearly completely inhibited by the MAO inhibitors at a concentration of 1 mM. SKF 525-A (1 mM) on the other hand had little influence on 3,4,5-TMPAA formation, but inhibited 3,4,5-TMBA formation to about 60 per cent, as can be seen in Table 4.

DISCUSSION

The oxidative degradation of mescaline to 3,4,5-TMBA, which is now established both *in vitro* and *in vivo*, is not proportional to the rate of mescaline deamination to 3,4,5-TMPAA. The highest rate of 3,4,5-TMBA formation was found with brain tissue preparations. *In vivo* also this pathway seems to be more important in brain than in visceral organs, as can be judged from the amounts of 3,4,5-TMBA in the different tissues after the injection of [2,6-^{3}H]/[8-^{14}C]mescaline [1].

The effect of SKF 525-A as an inhibitor, the activating effect of nicotinamide adenine nucleotides and the localization of the mescaline degrading enzyme in microsomal and nuclear fractions are consistent with the view that side chain degradation of mescaline is catalysed by an NAD(P)H dependent oxygenase or dehydrogenase. The activating effect of brain extracts on mescaline degradation suggest that additional factors are involved in the reaction.

Table 4. Inhibition of the formation of 3,4,5-TMBA and 3,4,5-TMPAA from mescaline by tranylcypromine, iproniazid, SKF 525-A, semicarbazide, thiosemicarbazide and disulfiram

Inhibitor	Net inhibition of oxidative deamination of mescaline (%)	Inhibition of 3,4,5-TMBA formation (%)	Inhibition of 3,4,5-TMPAA formation (%)
Tranylcypromine (trans-2-amino 1-phenyl-cyclopropane)	80 ± 2	37 ± 6	97 ± 3
Iproniazid (N_2-isopropyl-isonicotinic acid hydrazide phosphate)	76 ± 2	23 ± 1	98 ± 2
SKF 525-A [(2-diethyl-aminoethyl)-α,α-diphenylvalerate]	14 ± 4	61 ± 1	5 ± 3
Semicarbazide.HCl	0	0	0
Thiosemicarbazide.HCl	0	0	0
Disulfiram [bis-(diethyl-thio-carbamoyl)-disulfide]	0	0	0

Incubation mixture: 0·1 ml brain homogenate in 66 mM phosphate buffer pH 7·2 containing 19 mg brain tissue, 1 mM inhibitor and 5 mM [2,6-^{3}H]/[8-^{14}C]mescaline.HCl (9·2 μCi ^{3}H, 1·12 μCi ^{14}C). Before the addition of substrate the inhibitors were pre-incubated for 10 min at room temperature. Incubation conditions: shaking at 38° for 3 hr.

The values are means ± S.D. of three experiments.

As can be seen from Table 2 activation of brain extracts is not restricted to 3,4,5-TMBA formation. The deamination of mescaline to 3,4,5-TMPAA by nuclei is also enhanced considerably by phosphate buffer soluble constituents of brain tissue. This activation may be due to the content of aldehyde dehydrogenase of brain extracts [11]. In contrast to the nuclear fraction mitochondrial and microsomal fractions probably contain enough aldehyde dehydrogenase to yield maximal reaction rates. In this connection it should be noted that very high specific activities of MAO have been detected by Gorkin in the nuclear membrane [12], a finding which is consistent with the high rates of 3,4,5-TMPAA formation in the nuclear fraction.

Side chain degradation of mescaline closely resembles the degradation of amphetamine to benzoic acid, which is excreted *in vivo* as its glycine conjugate [13, 14]. The enzyme which is responsible for the first step of the reaction sequence, namely the degradation of amphetamine to phenylacetone, was characterized as an oxygen- and NADPH-dependent microsomal enzyme [3], which is inhibited *in vitro* [15] and *in vivo* [16] by SKF 525-A, These characteristics were repeatedly confirmed [14, 17–20]. A different characteristic was, however, reported by Mitra and Guha [21—23] who localized an "amphetamine dehydrogenase" in mitochondria and nuclei, which was equally capable of degrading amphetamine and mescaline as substrates [21]. (3,4,5-TMPAA was assumed, but not confirmed, to be the reaction product of mescaline in this reaction.)

Our findings on *in vitro* degradation of mescaline to 3,4,5-TMBA are similar to those of Axelrod on amphetamine degradation to phenylacetone, with some minor differences: high speed supernatant of liver extracts had a marked inhibitory effect on amphetamine degradation by liver microsomes [3], mouse liver extracts had no significant influence on mescaline degradation by brain microsomes or nuclei. Furthermore we found no significant difference in the activating effects with NADH and NADPH (see Table 3) in contrast to the findings with amphetamine as the substrate and liver microsomes as the enzyme source [3]. It is an open question therefore, whether the enzyme system responsible for mescaline degradation to 3,4,5-TMBA is identical with the amphetamine degrading system. In view of the low specificity of the microsomal oxygenases and dehydrogenases exact kinetic studies will be necessary to decide this question. It should be noted in this connection that all studies on the reaction mechanism of the oxidative deamination of amphetamine [9, 17–20] are concerned exclusively with the deamination of amphetamine to phenylacetone. In a second reaction sequence the propyl side chain is eliminated. However, this part of the reaction has not been studied. The formation of 3,4,5-TMBA comprises, by analogy, reaction steps which are most probably catalysed by different enzymes (or an enzyme system). The above mentioned differences between amphetamine and mescaline degradation may be due at least partially to differences in the later steps, not necessarily in the first step of the reaction sequence. The cationic mescaline metabolites, which were found in tissue after the injection of radioactive mescaline [1] are probably intermediates of the transformation of mescaline to 3,4,5-TMBA.

One of the prerequisites for side chain degradation of arylalkylamines seems to be a poor substrate property for MAO. Substrates of MAO, such as tyramine, benzylamine and phenylethylamine were, if at all, only very slowly degraded by the microsomal enzyme [3]. Amphetamine is, on the other hand, a competitive inhibitor of MAO [24]; mescaline is slowly deaminated by MAO, both *in vitro* [10, 25] and *in vivo* [26, 27], In contrast to mescaline its non-hallucinogenic isomer, 2,3,4-trimethoxy-β-phenylethylamine is deaminated by MAO at a high rate [10, 26], and only small amounts of 2,3,4-TMBA were found in urine after the injection of this compound, as compared with 3,4,5-TMBA after mescaline administration [28].

It is evident from many studies [29–33] that the amino group at 8-C is a feature for psychotomimetic properties, furthermore the electron donor–acceptor properties are essential. Mescaline fulfills these criteria. Nevertheless there are numerous findings which indicate that metabolic steps may be inserted between resorption and the display of the hallucinogenic activity [34–36]. Shulgin [29] has shown that the enantiomers of the amphetamines with R configuration at 8-C (and the R 5-C enantiometer of LSD) are hallucinogenic, whereas the isomers of these compounds with S configuration do not show psychotomimetic properties. The first step of the microsomal oxidation of amphetamine *in vitro* is 8-C [17, 19] or *N*-oxidation [20]. An oxidative attack of 8-C of mescaline would cause the formation of a chirality center. The study of the mechanism of the oxidative degradation of mescaline to TMBA could form a basis for the disclosure of the assumed relationship between the hallucinogenic properties of mescaline and its metabolism.

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