Biochemical Pharmacology, Vol. 26, pp. 1757–1762. Pergamon Press, 1977. Printed in Great Britain.

STEREOCHEMICAL COURSE *IN VIVO* OF ALPHA-METHYLDOPA DECARBOXYLATION IN RAT BRAINS*

MATTHEW M. AMES,† KENNETH L. MELMON and NEAL CASTAGNOLI, JR.‡

Division of Clinical Pharmacology, Departments of Pharmaceutical Chemistry, Medicine, and Pharmacology, and the Cardiovascular Research Institute, University of California, San Francisco, CA 94143, U.S.A.

(*Received* 16 *August* 1976; *accepted* 29 *December* 1976)

Abstract—The metabolic fate *in vivo* of the antihypertensive agent α-methyldopa (α-MD) has been examined in rat brain tissues with the aid of ^{13}C- and ^{2}H-labeled compounds and chemical ionization mass spectrometry. After intraperitoneal administration of (S)-α-MD, endogenous catecholamines in the caudate nucleus and hypothalamus were replaced by the corresponding α-methylated compounds. In contrast to these results, brain catecholamines were unaffected when (R)-α-MD was given. Transport of the amino acids into the brain was found to be highly stereoselective for the (S)-enantiomer. After direct administration of (S)- or (R)-α-MD into rat brain (third ventricle), evidence of metabolism was obtained only for the pharmacologically active (S)-enantiomer. These results are consistent with the hypothesis that the centrally mediated antihypertensive properties of (S)-α-MD are dependent on its metabolic conversion in the brain to α-methyldopamine and/or α-methylnorepinephrine.

Extensive studies on the pharmacologic properties of the clinically useful [1–6] antihypertensive agent α-methyldopa (α-MD, I§) have led to a "central false neuro-transmitter" proposal to describe its mode of action [7–10]. The fundamental biochemical events that underlie this mechanism are active transport of the amino acid into the brain followed by enzymatic decarboxylation to α-methyldopamine (α-MDA, II) and oxidation of II to α-methylnorepinephrine (α-MNE, III). The antihypertensive activity [2] and catecholamine-depleting properties [11] of α-MD are associated with the (S)-enantiomer, (S)-I. Additionally, evidence based on the excretion of α-MDA [12–15] and assays with partially purified enzyme preparations [16] suggest that the stereospecificity of the antihypertensive activity of α-MD is related to the decarboxylation of the amino acid by (S)-DOPA decarboxylase. Recent studies, however, have indicated that brain [17] and other tissues [18, 19] contain decarboxylases other than (S)-DOPA decarboxylase with undefined sterochemical substrate requirements. In order to further characterize the structural features associated with the metabolic disposition of α-MD, we have examined the metabolism of (S)-α-MD [(S)-I], (R)-α-MD[(R)-I], and (R,S)-α-MD in rats. With the aid of stable isotopically labeled compounds (see below) and a chemical ionization (c.i.) mass spectrometric assay for endogenous catecholamines and α-methylated catecholamines [20], we have investigated the stereochemical requirements for the decarboxylation of α-MD *in vivo* in the anatomically well-defined, catecholamine-rich caudate nucleus and hypothalamus. As part of these studies, we also have examined the stereochemical requirements for transport of the (S)- and (R)-amino acids into the brain and the fate of (S)- and (R)-α-MD administered directly into the rat brain via a chronically implanted cannula terminating in the third ventricle.

MATERIALS AND METHODS

[^{13}C]α-MD (89 per cent isotopic enrichment), specifically labeled at the benzylic carbon atom, was synthesized as previously described for the 65 per cent enriched compound [21]. Resolution of the ^{13}C-labeled amino acid was accomplished by modification of a literature procedure [22, 23]. The amino acid hydrochloride (2.53 g, 10.2 m-moles) was heated under reflux and a nitrogen atmosphere in acetic anhydride–pyridine (11 ml each) for 13 hr. After complete removal of the acetic anhydride and pyridine, the residue was treated with 2 N HCl (4 ml) and the resulting mixture extracted with ethyl acetate (4 × 50 ml). The combined extracts were washed with water (2 × 25 ml), dried (Na_2SO_4), and concentrated to yield after crystallization from acetone the white *tris*–acetyl derivative (1.85 g, 54 per cent): m.p.‖ 191–93° (lit. [23] m.p. 197–99°); nmr (pydidine-d_5) δ 7.43 to 7.02 (m, 3H, ArH), 3.99 and 3.58 (AB q, 2H, J = 18.8 Hz, Ar-$^{12}CH_2$), 3.99 and 3.58 (d of AB q,

* This investigation was supported in part by National Institutes of Health Grant GM 16496 and Training Grant 3920.

† Present address: Section on Biochemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, MD 20014.

‡ Request for reprints to be sent to: Dr. Neal Castagnoli, Jr., Department of Pharmaceutical Chemistry, School of Pharmacy, 926-S, University of California, San Francisco, CA 94143.

§ See Fig. 1 for chemical structures.

‖ Solvents were removed on a rotary evaporator under vacuum. Melting points were taken on a Thomas–Hoover apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a Varian A-60A instrument. Chemical ionization mass spectra were taken on an Associated Electronics Inc. model MS 902 double focus mass spectrometer equipped with a direct inlet system and modified for chemical ionization mass spectrometry. The reagent gas was isobutane at a pressure of 0.5 to 1.0 Torr, at the indicated source temperature.

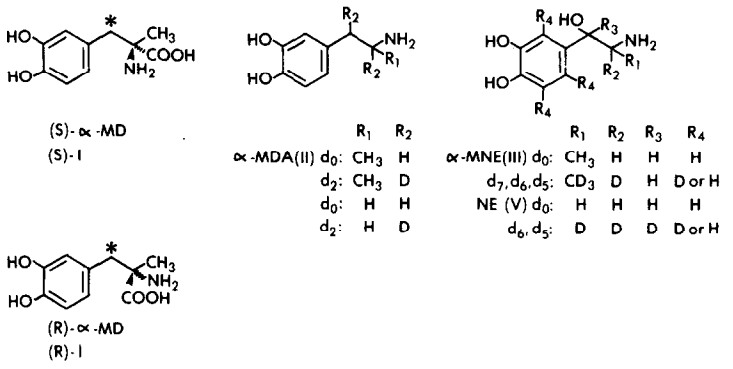

Fig. 1. Structures of substrates, internal standards and metabolites described in text. The ^{13}C label is located at the benzylic carbon atom, designated by an asterisk.

2H, $J_{^{13}CH}$ = 132 Hz, Ar-$^{13}CH_2$), 2.23 (s, 3H, $COCH_3$), 2.20 (s, 3H, $COCH_3$), 2.04 (s, 3H, $NCOCH_3$), 1.69 (s, 3H, $Ar^{12}CCCH_3$) and 1.69 (d, 3H, $J_{^{13}CCH}$ = 3.5 H_δ, $Ar^{13}CCCH_3$); c.i.m.s. (280°) m/e (rel. int.) 339 (100, MH^+), 338 (11), 321 (89). An acetone (30 ml) solution of the resulting (R,S)-*tris*-acetyl derivative (2.96 g, 8.76 m-moles) and quinine (3.35 g, 10.3 m-moles) was stirred and then maintained at 0–5° for 3 hr. The solid quinine salt of this *tris*-acetyl derivative [1.85 g, 64 per cent, m.p. 155–170° (lit. [23] m.p. 164–66°)] in water (4 ml) and 2 N HCl (6 ml) was extracted with ethyl acetate (4 × 20 ml). The combined extracts were washed (8 ml of 2 N HCl), dried ($MgSO_4$), and evaporated to yield 0.9 g (100 per cent) of the resolved acid: m.p. 178–79° (lit. [23] m.p. 181–83°); $[\alpha]_{589}^{25}$–64.86° (C 2, 96% ethanol) (lit. [23] $[\alpha]_{589}^{25}$–74.5°, C 2, 96% ethanol). The low rotation led us to subject this material to the resolution with quinine a second time: m.p. 177–79°; $[\alpha]_{589}^{25}$–68.77° (C 2, 96% ethanol). Hydrolysis of the resolved *tris*-acetyl derivative (1.39 g, 4.13 m-moles) in refluxing 6 N HCl (35 ml) gave, after removal of solvent, the extremely hygroscopic hydrochloride salt of (S)-α-MD[^{13}C] [(S)-I[^{13}C]·HCl, 0.97 g, 95 per cent]. Working under oxygen-free nitrogen, the amino acid hydrochloride (0.97 g, 3.9 m-moles) in water (2 ml) was treated with triethylamine (0.6 ml, 4.3 m-moles). After cooling for 3 hr in an ice-water bath, the solids were collected by suction filtration, washed once with water (3 ml), and dried *in vacuo* to yield (S)-α-MD[^{13}C] [(S)-I[^{13}C], 0.72 g, 87 per cent]: m.p. 290–300°, dec. (lit. [23] m.p. 290–300°, dec.). The nmr spectrum was identical to the reported spectrum of I[^{13}C] (65 per cent enrichment [23]) except for effects due to the higher ^{13}C enrichment. The enantiomeric purity of (S)-I[^{13}C] was established to be 98 per cent by observing only one peak on gas–liquid chromatography (g.l.c.) analysis of the drastereomeric amide formed from *tris-O*-methylated α-MD[^{13}C] and (−)-1-α-methoxy-α-trifluoromethylphenylacetyl chloride.* Racemic I shows two well-resolved peaks when analyzed by this assay.

Syntheses of the deuterium-labeled internal standards dopamine-d_2 (DA-d_2, IV-d_2) and (R,S)-α-MDA-d_2 (II-d_2) were achieved by following the previously reported synthesis of α-MDA [12] except that lithium aluminium deuteride (> 99 per cent d) replaced the lithium aluminum hydride. Syntheses of the deuterium-labeled internal standards (R,S)-norepinephrine-d_6,d_5 (NE-d_6,d_5, V-d_6,d_5) and (R,S)-erythro-α-MNE-d_7,d_6,d_5 (III-d_7,d_6,d_5) have been published [24]. The "pseudoracemic mixture" of α-MD was prepared by mixing equal amounts of (R)-α-MD and (S)[^{13}C]-α-MD.

Animal procedures. Male Wistar rats (300–400 g) were used in all experiments. The various forms of α-MD (30 mg/ml in 0.9% NaCl–0.1 N HCl) were given intraperitoneally (i.p., 200 mg/kg) or intraventricularly (i.vt., 1 mg/kg), the latter under light ether anesthesia via a 22 gauge stainless steel cannula. The cannula had been stereotaxically implanted under pentobarbital anesthesia (50 mg/kg) into the third ventricle 24 hr prior to α-MD treatment. The animals were killed by decapitation either 5 hr (for catecholamine determinations) or 1 hr (for amino acid determinations) after i.p. drug administration. The animals treated i.vt. were sacrificed after 2 hr. In all experiments the caudate nucleus and hypothalamus were quickly isolated, weighed and immediately frozen at −70° until analyzed.

Analytical procedures. A stock solution of internal standards (DA-d_2, α-MDA-d_2, NE-d_6,d_5, and α-MNE-d_7,d_6,d_5) was prepared in 0.1 N HCl and was standardized against a solution of the corresponding unlabeled amines (10 μM in each compound) by the c.i. mass spectral assay described below. The stock solution concentrations of the internal standards were adjusted such that the ions monitored for NE-d_6, α-MNE-d_7, DA-d_2, and α-MDA-d_2 were equal in intensity to the corresponding ions of the d_0-amines. A second standard solution of (R,S)-α-MD[^{13}C] (10 μM) was prepared in 0.1 N HCl. The tissues were homogenized with a Teflon–glass Potter–Elvehjem tissue homogenizer (six to eight strokes) in 5% trichloroacetic acid (3 ml) containing 0.5% sodium metabisulfite (an antioxidant) to which the deuterated amine stock internal standard solution (200 μl for the caudate nucleus and 100 μl for the hypothalamus) or [^{13}C]amino acid internal standard solution (500 μl) had been added.

For amine analysis, the pH of the homogenates was adjusted to 4.0 with 0.5 M acetic acid–sodium acetate (pH 5), and the resulting solutions were centrifuged (15,000 *g* for 15 min). The supernatant fractions were

* J. Gal and M. Ames, unpublished observations.

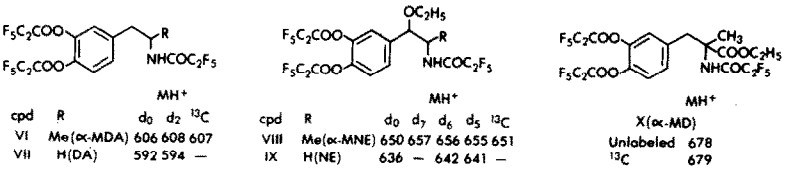

Fig. 2. MH^+ values for various derivatives monitored in c.i. mass spectra.

applied to colums packed with the sodium form of BioRad AG 50W-x8 cation exchange resin (0.5 cc),* and the columns were eluted sequentially with 0.5 M acetic acid–sodium acetate (pH 5, 5 ml), 0.1 N HCl (10 ml), anhydrous ethanol (10 ml), and anhydrous 2 N ethanolic HCl (8 ml). This last fraction, which, according to recovery studies, contained over 90 per cent of the amines of interest, was evaporated to dryness under vacuum in the cold in a Concentratube,† and the residue in anhydrous 1 N ethanolic HCl (0.25 ml) was capped and heated for 1 hr at 60–70° (oil bath) to convert α-MNE and NE to their corresponding β-ethyl ethers. The solvent was removed under a stream of dry nitrogen, and the resultant residues were treated with pentafluoropropionic anhydride (PFPA, 0.25 ml) at 60° for 20 min in capped tubes to form the pentafluoropropionyl (PFP) derivatives VI (from α-MDA), VII (from DA), VIII (from α-MNE) and IX (from NE), respectively, shown in Fig. 2. The reaction mixtures were then concentrated under nitrogen to *ca.* 10 μl for c.i. mass spectral analysis.

Analysis for the amino acids proceeded in a similar fashion except that the pH of the homogenates was not adjusted and the hydrogen form of the resin was used. The sequential elutions consisted of 0.1 N HCl (10 ml), anhydrous ethanol (10 ml) and 2 N ethanolic HCl (10 ml). The final eluent contained the amino acids of interest and was concentrated to dryness *in vacuo* in the cold in a Concentratube. The residue in 2 N ethanolic HCl (0.25 ml) was heated for 6 hr at 60° to form the ethyl ester. The solvent was removed under a stream of dry nitrogen, and the residue was treated with PFPA as described above to form the PFP derivative X in preparation for c.i. mass spectral analysis.

The concentrated PFPA solutions were applied to the ceramic tip of an AEI MS 902 direct insertion probe. Spectra were recorded at 170–180° with isobutane (0.7 Torr) as reagent gas. Each sample was scanned over the relevant mass region three to five times and the appropriate ion current intensity lines (Fig. 2) were averaged arithmetically (only when 10 mm above background levels). In general, ion current intensity ratios varied less than 5 per cent. Those samples that did not provide sequential ion intensity ratios within 5 per cent were discarded.

RESULTS

Typical c.i. mass spectral scans of the derivatized, deuterated internal standards and corresponding catecholamines found in the caudate nucleus isolated from the brains of rats treated with (S)-α-MD and (R)-α-MD are given in Fig. 3b and 3c respectively. Figure 3a presents the mass spectrum of a mixture of the eight pure amine standards carried through the analytical procedure. Ion intensities for the derivatized, deuterated internal standards for α-MNE appear at m/e 657, 656 and 655 (MH^+ for VIII d_7,d_6,d_5), for NE at m/e 642 and 641 (MH^+ for

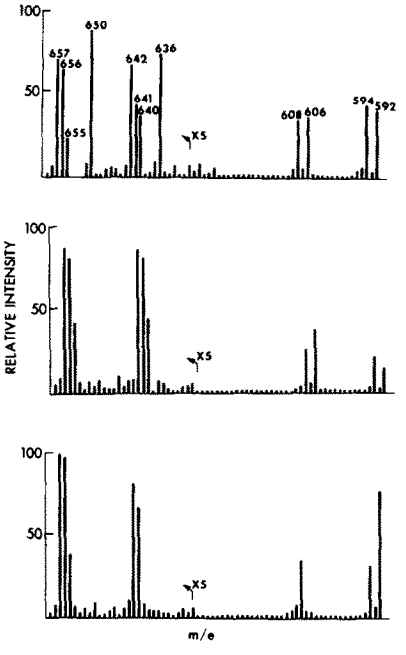

Fig. 3. Chemical ionization mass spectra of derivatized catecholamines, α-methylcatecholamines and their deuterated internal standards. Upper panel (a): mixture of derivatized standard amines; center (b): from rat brain caudate nucleus after i.p. (S)-α-MD; and lower (c): from rat brain caudate nucleus after i.p. (R)-α-MD. The mass numbers corresponding to the MH^+ ions of the derivatized catecholamines are as follows: m/e 657 (α-MNE-d_7), 650 (α-MNE-d_0), 642 (NE-d_6), 636 (NE-d_0), 608 (αMDA-d_2), 606 (αMDA-d_2), 594 (DA-d_2), 592 (DA-d_0).

* All resins were conditioned prior to use by washing the resin (50 g) sequentially with 500 ml each of water, 1 N NaOH, water, 2 N HCl, and water. The sodium form of the resin (0.5 ml, on column) was prepared by sequential washing with 2 N HCl (10 ml), water (until Cl^- free by $AgNO_3$ test), 1 N NaOH (10 ml) and water (until pH 7). The hydrogen form of the resin was prepared by washing the resin (0.5 ml, on column) with 2 N HCl (10 ml) followed by water (until Cl^- free).

† Concentratubes were obtained from Laboratory Research Co., P.O. Box 36509, Los Angeles, CA 90036.

Table 1. Levels of dopamine and α-methyldopamine in the rat brain caudate nucleus in control animal and after administration of (S)-, (R,S)-, and (R)-α-methyldopa, and (S)-α-methyldopa[^{13}C]*

Drug given i.p. or [i.vt.]	Dopamine (nmoles/g)	α-Methyldopamine (nmoles/g)
(S)-α-MD	34	46
(S)-α-MD	15	34
(S)-α-MD[^{13}C]	21	82
(R,S)-α-MD	44	54
(R,S)-α-MD	51	57
(R)-α-MD	66	<2
(R)-α-MD	62	<1
Control	42	<1
[(S)-α-MD]	33	23
[(R)-α-MD]	52	<5

* In all i.p. experiments, 200 mg/kg were given; for the i.vt. studies, 1 mg/kg was given. In the control i.p. experiment, vehicle (0.9% NaCl–0.1 N HCl) only was given.

IX-d_6,d_5),* for α-MDA at m/e 608 (MH^+ for VI-d_2) and for DA at m/e 594 (MH^+ for VII-d_2). Ion intensities at m/e 650 (MH^+ for VIII-d_0), m/e 636 (MH^+ for IX-d_0), m/e 606 (MH^+ for VI-d_0) and m/e 592 (MH^+ for VII-d_0) correspond to the undeuterated amines α-MNE, NE, α-MDA, and DA respectively.†

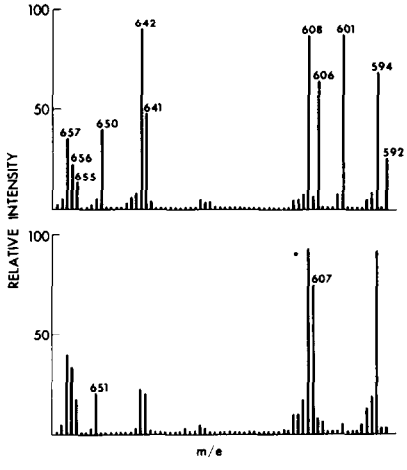

Fig. 4. Chemical ionization mass spectra of rat brain hypothalamus amines and deuterated internal standards after i.p. administration of (S)-α-MD [(a) upper panel] and (S)-α-MD[^{13}C] [(b) lower panel]. Note that the MH^+ ions corresponding to the derivatized α-MDA (m/e 606) and α-MNE (m/e 650) are shifted by one atomic mass unit to m/e 607 and 651 when (S)-α-MD[^{13}C] is given. The intense ion at m/e 601 appeared irradically in hypothalamic studies and has not been identified.

* The ion located at m/e 640 is derived from IX-d_4 formed from exchange of an aromatic deuterium atom of IX-d_6,d_5 during work-up. Since in this study we did not attempt quantitative estimations of NE and α-NME, we have not corrected for this loss of label. Analogous loss of the deuterium atoms present in the DA and α-MDA internal standards is not possible since these atoms are sp^3 bonded to carbon.

† In order to simplify the discussion that follows, reference will be made to the parent amines even though the mass spectral ions cited will actually be the appropriate derivative.

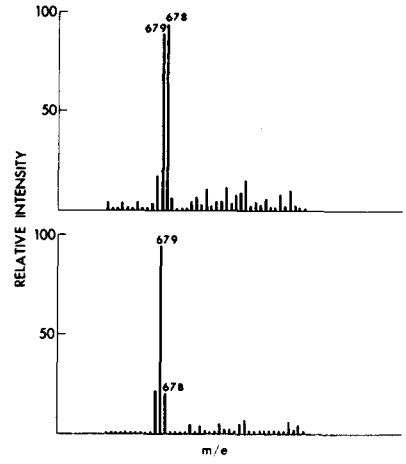

Fig. 5. Chemical ionization mass spectra of caudate nucleus extracts worked up for amino acids. Figure 5a (upper panel) was obtained after i.p. administration of (S)-α-MD. Figure 5b (lower panel) was obtained after i.p. administration of (R)-α-MD. The ions observed at m/e 679 in both spectra are derived from the α-MD[^{13}C] internal standard. The ion at m/e 678 is derived from the administered (S)-α-MD[^{13}C]. Both tracings are corrected for the 11% ^{12}C contaminant in the ^{13}C internal standard.

Consistent with literature reports [25], NE (m/e 636) and α-MNE (m/e 650) were not detected in the caudate nucleus tissues. In the samples obtained from (S)-α-MD-treated animals (Fig. 3b), ion current intensities at m/e 592 and 606 (DA and α-MDA) were comparable to the mass lines at m/e 594 and 608 (deuterated internal standards). Similar tracings were obtained after (R,S)-α-MD and after (S)-α-MD[^{13}C], except that in the latter case the ion corresponding to α-MDA[^{13}C] (m/e 607) replaced the m/e 606 ion. The c.i. mass spectral tracing obtained from the (R)-α-MD-treated animal (Fig. 3c) shows a much more intense ion at m/e 592 (DA) and essentially background current at m/e 606 (α-MDA). When the pseudoracemic mixture composed of equal amounts of (R)-α-MD and (S)-α-MD[^{13}C] was given, the ion current intensity at m/e 607 [α-MDA[^{13}C] derived from (S)-α-MD[^{13}C]) was equal to that of the internal standard at m/e 608 (α-MDA-d_2). Ion current at m/e 606 [α-MDA derived from (R)-α-MD] when corrected for the 11 per cent ^{12}C content in the (S)-α-MD[^{13}C] was essentially the same as background. The quantitative estimations of α-MDA and DA in the caudate after i.p. administration of various forms of α-MD are summarized in Table 1. Too few analyses were carried out for statistical treatment of these data. However, the stereochemical dependence for formation of α-MDA is clearly evident.

Results obtained from samples of the hypothalamus (Fig. 4a) were similar to the results from samples of the caudate except that ions corresponding to the derivatives of both α-MDA (m/e 606) and α-MNE (m/e 650) were readily detected, but again only after (S)-α-MD treatment. Consistent with these assignments were the values obtained from c.i. mass spectra of the hypothalamic amines isolated from an animal treated i.p. with (S)-α-MD[^{13}C]. The ions assigned to the α-MDA and α-MNE derivatives VI and VIII

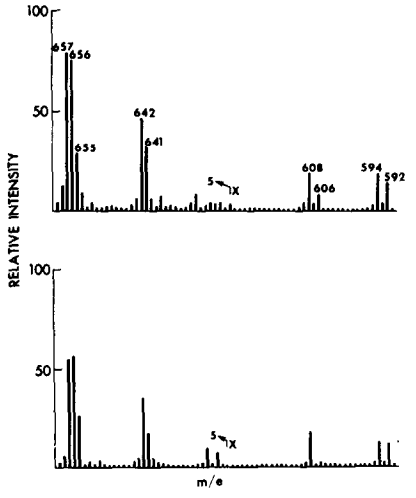

Fig. 6. Chemical ionization mass spectra of derivatized catecholamines, α-methylcatecholamines and their deuterated internal standards, found in the rat caudate nucleus. Upper panel (a) after intraventricular administration of (S)-α-MD, and lower panel (b) after intraventricular administration of (R)-α-MD.

have shifted from m/e 606 to 607 and 650 to 651, respectively (Fig. 4b). After (R)-α-MD, hypothalamic NE levels were the same as control levels, and no significant ion currents were observed at m/e 606 and 650, even though the deuterated internal standard derivatives gave rise to prominent ions. As a final check, the pseudoracemic mixture was given. The mass spectral tracing of the hypothalamic amines was similar to that shown in Fig. 4b; the α-MDA and α-MNE found were derived solely from (S)-α-MD[^{13}C].

Our failure to detect α-MDA and α-MNE after i.p. administration of (R)-α-MD led us to examine levels of the (R)- and (S)-amino acids in the caudate nucleus. In these experiments, (R,S)-α-MD[^{13}C] served as an internal standard, which, as its ethyl ester–PFP derivative (X[^{13}C]), appears in the c.i. mass spectrum at m/e 679. The presence of α-MD (*ca.* 100 nmoles/g) in the caudate after administration of the (S)-enantiomer is indicated clearly by the intense ion appearing at m/e 678 (Fig. 5a). A similar tracing was obtained after administration of (R,S)-α-MD. However, when corrected for the ^{12}C content of the ^{13}C internal standard (11 per cent), the ion intensity at m/e 678 after administration of (R)-α-MD was found to be very low (Fig. 5b). Results similar to those with (S)-α-MD[^{13}C] were obtained with the pseudoracemic mixture of amino acids. Based on these data, we concluded that transport of α-MD into the brain is probably a highly stereoselective process involving mechanisms analogous to the active transport of endogenous aromatic amino acids [26].

In order to bypass the blood–brain stereochemical barrier, (S)- and (R)-α-MD were administered directly into the brain via a cannula implanted into the third ventricle. The caudate nucleus once again was analyzed for catecholamines. As depicted in Fig. 6a [(S)-α-MD] and Fig. 6b [(R)-α-MD] and summarized in Table 1, α-MDA was detected in the tissue isolate only after (S)- but not (R)-α-MD was given.

DISCUSSION

The results summarized in this paper are consistent with the contention that the antihypertensive action of α-MD is linked to its metabolic conversion to α-MDA and α-MNE in the brain. Comparatively high levels of α-MDA in the caudate nucleus (Table 1) and both α-MDA and α-MNE in the hypothalamus were found only after administration of (S)- and (R,S)-α-MD. Additionally, endogenous levels of DA and NE were lower than the control levels. The pharmacologically inactive (R)-α-MD did not show analogous biochemical properties. After i.p. administration of the (R)-enantiomer, the levels of endogenous amines were unchanged in the caudate nucleus and hypothalamus. There was no evidence to indicate the presence of the α-methylated catecholamines in these tissues. The administration of pseudoracemic α-MD [(R)-[^{12}C]: (S)-[^{13}C]] allowed us to study the stereoselectivity of decarboxylase activity *in vivo* on both enantiomers of α-MD, simultaneously and in a single animal. Brain α-MDA and α-MNE were derived exclusively from the (S)-α-MD[^{13}C].

These results could be explained either by the selective transport of the (S)-α-MD but not the (R) across the blood-brain barrier as suggested by the relatively high levels of (S)-α-MD vs (R)-α-MD found in the caudate nucleus or the lack of central decarboxylase activity for transported (R)-α-MD. This issue was clarified by directly administering the two enantiomers into the third ventricle of rat brains. Only the pharmacologically active (S)-isomer led to production of α-MDA in the caudate. On the basis of these experiments coupled with less direct evidence [11–15], it may be concluded that, in mammals, α-MD interacts with macromolecules in a stereospecific fashion analogous to naturally occurring substrates both in terms of active transport mechanisms and enzymatic conversion.

Acknowledgements—The authors wish to thank Dr. Martha Hamlet for preparing the animals for the intraventricular studies and are grateful to Merck, Sharp & Dohme for providing unlabeled (S)-, (R)-, and (R,S)-α-MD.

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