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IRREVERSIBLE INHIBITION OF AROMATIC-L-AMINO ACID DECARBOXYLASE BY α-DIFLUOROMETHYL-DOPA AND METABOLISM OF THE INHIBITOR

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Abstract— *In vitro*, α-difluoromethyl DOPA (DFMD, RMI 71801), at concentrations from 2 to 20 μM, inhibits aromatic-L-amino acid decarboxylase (AADC) in a time-dependent manner. After inhibition, the activity of the enzyme cannot be restored by dialysis. The inhibition is prevented by addition of an excess of the substrate L-DOPA. Near to one mole of ring-tritiated DFMD binds to one mole of enzyme during the inhibition process. The absorption spectrum of AADC is slightly modified by DFMD. No transformation product of the inhibitor is accumulated during incubation with the enzyme. It is concluded that DFMD is an enzyme-activated inhibitor of AADC. *In vitro* and *in vivo*, DFMD is a substrate of catechol *O*-methyltransferase.

We have recently reported that two newly synthesized DOPA analogues, α-acetylenic DOPA and α-vinyl DOPA, competitively inhibit aromatic-L-amino acid decarboxylase (EC 4.1.1.26, AADC) from hog kidney [1], but inhibition appeared to have also an irreversible component. *In vivo*, these compounds inhibit the decarboxylation of exogenously-supplied L-DOPA and 5-hydroxytryptophan in the periphery with a consequent short-lasting elevation of brain catecholamines and serotonin.

Two other irreversible inhibitors of AADC have been reported to be more potent and to have longer durations of action *in vivo* than the two compounds above. These inhibitors are α-difluoromethyl DOPA (DFMD) [2] and α-monofluoromethyl DOPA (MFMD) [3]. Only MFMD was potent enough to reduce synthesis of biogenic amines in the periphery and in the brain [3]. MFMD did not show preferential peripheral effects at doses higher than 5 mg/kg, while DFMD was selective in its action in the periphery at 500 mg/kg. DFMD might therefore be used in combination with L-DOPA to increase the synthesis of dopamine in the brain.

This paper provides direct evidence that DFMD inhibits AADC by a mechanism previously proposed by Palfreyman *et al.* [2] and describes the *in vitro* and *in vivo* metabolism of the compound. During the course of these experiments Kollonitsch *et al.* [4] reported that the same mechanism is valid for MFMD.

MATERIALS AND METHODS

Chemicals. DFMD was synthesized in our laboratories [5]. [Ring 2,5,6-^{3}H]DFMD was synthesized by the "Commissariat à l'Energie Atomique" in Saclay, France. The synthesis was done by tritium exchange and the specific activity was 0.7 Ci/mmole. [1-^{14}C]L-DOPA, 1–5 mCi/mmole, and [ring 2,5,6-^{3}H]DOPA, 20–40 Ci/mmole, were purchased from the Radiochemical Center (Amersham, U.K.). All other compounds were of analytical grade and commercially available.

Enzymes. AADC was purified from hog kidney and its activity was determined as previously described [1]. The specific activity of the purified enzyme (7500–8000 units/mg) was in the range of that obtained by Christenson *et al.* [6] for the enzyme purified to homogeneity. The enzyme was kept at 0°. It lost about 50 per cent of its activity in six weeks. Specific activity was determined at the time of each experiment by the CO_2 trapping method described by Christenson *et al.* [6]. One unit of activity is defined as the total amount of enzyme which produces 1 nmole of CO_2 per min with [1-^{14}C]L-DOPA as substrate.

Catechol *O*-methyltransferase was partially purified according to the method of Axelrod [7]. For determination of activity, 100 μl enzyme preparation was incubated in 10 mM phosphate buffer, pH 7.4, with 70 μM *S*-adenosyl-L-methionine, 125 μM $MgCl_2$, 100 μM [^{3}H]DFMD (100 μCi) in a final volume of 1 ml at 30°.

The reaction was stopped by addition of 500 μl of 5 M acetic acid and the mixture was chromatographed on cellulose t.l.c. plates using butanol–acetic acid–water (60 : 20 : 20) as solvent. The radioactivity of the separated compounds was determined with a t.l.c. plate radioactivity scanner (Dünnschicht-Scanner II from Berthold, Wildbad, F.R.G.).

Time-dependent inhibition of AADC. To 100 μl of 50 mM phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol, were added 50 μl of an aqueous solution of the inhibitor and 50 μl of enzyme solution. The mixture was incubated at 37° and 20 μl aliquots were withdrawn to measure AADC activity at the times indicated.

Binding of [*ring*-2,5,6-^{3}H]*DFMD to AADC*. Ten millimolar phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol and 20 μM [^{3}H]DFMD (0.7 Ci/mmole) and enzyme (0.15 mg protein/ml) in a final volume of 1 ml was incubated at 30°. Aliquots (100 μl) were withdrawn at various times after the addition of enzyme. These aliquots were mixed with 100 μl of bovine serum albumin (2 mg/ml). Then 20 μl of 0.3 M trichloroacetic acid (TCA) were added and

the tubes were kept in ice for 30 min. The mixtures were filtered on a 0.45 μm millipore filter and washed with 50 ml 0.3 M TCA. The filters were solubilized by incubation for 48 hr in 1 ml soluene (Packard) and 0.5 ml H_2O. The radioactivity was measured by liquid scintillation spectrometry employing Dimilume (Packard) as scintillation medium. Results were corrected for quenching. The time-dependent inhibition of AADC during the binding of DFMD was determined in conditions identical to those indicated above.

Absorption spectrum of AADC. The spectrum of an AADC solution containing 10 mM phosphate buffer, pH 7.2, and 5 mM mercaptoethanol, was recorded with a spectrophotometer Acta III from Beckman Instruments Inc. (Fullerton, CA). DFMD was then added in excess, its concentration being about 10-fold that of the enzyme. The spectrum of the mixture was recorded at different times after mixing.

Metabolism of DFMD by AADC. DFMD was measured in incubation mixtures by high performance liquid chromatography (h.p.l.c.) as follows: to 850 μl of 50 mM phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol, 100 μl of 2 mM DFMD and 50 μl of enzyme (40 μg protein) were added. Where indicated, pyridoxal phosphate (PLP) was 50 μM. After various times of incubation at 20°C, 100 μl aliquots were mixed with 20 μl of 0.6 M TCA. Following centrifugation, aliquots of the TCA solution (10 μl) were directly injected into a 30 cm × 3.9 mm μBondapak C_{18} column from Waters Assoc. (Milford, MA). Elution with 0.02 M acetic acid was achieved at a flow rate of 1 ml/min. A waters model 204 liquid chromatograph, consisting of a 6000A solvent delivery system, a U6K universal injector and an u.v. absorbance detector, model 440, was employed. The absorbance of the eluant was monitored at 280 nm.

Condensation of DFMD and L-DOPA with pyridoxal phosphate (PLP). The products of condensation between DFMD or L-DOPA with PLP were obtained by mixing under nitrogen equimolar amounts of aqueous solutions of both compounds, at pH 7.0 and 37°, for 15 hr. Using [^{3}H]DFMD, under similar conditions, the formation of a new compound was detected on cellulose t.l.c. plates.

Both products were then precipitated by a slow addition of 5 M HCl at 0°. The compounds were redissolved in the minimum amount of water and reprecipitated by adding 5 M HCl. The same procedure was repeated a third time. The purity of the compounds was checked by h.p.l.c. by monitoring the eluates by both u.v. and electrochemical detection (model LC 15 detector from Bioanalytical Systems, West Lafayette, IN) [8].

Metabolism of [^{3}H]DFMD. [Ring-2,5,6-^{3}H]DFMD was administered orally to overnight fasted male rats (Sprague–Dawley, 150–200 g) at the dose of 100 mg/kg, 10 mCi/kg. The rats were then housed for various times in metabolic cages and the urine was collected in flasks surrounded by dry ice. Urine aliquots (10–100 μl) were directly applied on t.l.c. cellulose plates for separation of metabolites from the original drug. Tissue metabolites were extracted by homogenization of the organs (brain, liver or kidney) in 10 vol. water. The homogenates were centrifuged at 35,000 *g* for 30 min, then deproteinized by filtration with an immersible molecular separator (Millipore). The ultrafiltrate was concentrated by lyophilization and then chromatographed on cellulose t.l.c. plates.

In a separate experiment, [^{3}H]DFMD was administered orally to rats as described above and after various times the rats were decapitated and samples (0.5 g or less) of brain and liver were lyophilized. The radioactivity of the dry tissues and of the water recovered by lyophilization was determined with a sample oxidizer IN 1401 from Intertechnique (Plaisir, France). For comparison, *in vivo* tritium exchange was also determined following oral administration of [ring-2,5,6-^{3}H]DOPA.

RESULTS

Time-dependent inhibition of AADC by DFMD. Incubation of AADC with DFMD resulted in a time-dependent loss of enzyme activity, as shown in Fig. 1. At 10 μM DFMD the enzyme lost 50 per cent of its activity in about 10 min.

In order to determine if the observed inhibition was irreversible, the enzyme, incubated for 30 min with DFMD at various concentrations from 2 to 100 μM, was extensively dialysed. The experimental details and the data obtained are shown in Table 1. After 24 hr dialysis there was no recovery of activity compared to a control preparation similarly dialysed. After 48 hr dialysis there was a partial recovery of activity only at 2 and 10 μM DFMD. It is therefore concluded that the enzyme inhibitor complex is not readily dissociable.

When inhibition of AADC by DFMD was studied in the presence of 4 mM L-DOPA, the inhibitory effect was markedly attenuated (Fig. 2).

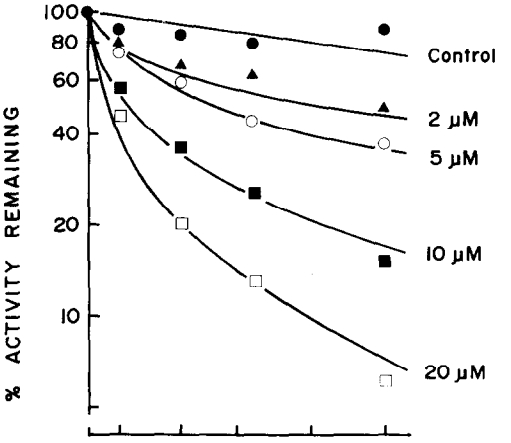

Fig. 1. Time-dependent inhibition of hog kidney aromatic L-amino acid decarboxylase (AADC) by α-difluoromethyl DOPA (DFMD). AADC (78 units) was incubated (37°) with various concentrations of DFMD in 50 mM phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol in a total volume of 200 μl. At the times indicated, 20 μl aliquots were withdrawn to measure enzyme activity (4972 units per mg protein at 0 time).

Table 1. Effect of dialysis on the activity of aromatic-L-amino acid decarboxylase inhibited by α-difluoromethyl DOPA (DFMD)*

DFMD (μM)	Aromatic-L-amino acid decarboxylase activity (% of activity of control)		
	Before dialysis	After 24 hr dialysis	After 48 hr dialysis
0	100	100	100
2	50	46	77
10	8	5	11
50	3	2	9
100	2	0	3

* The incubation mixtures (1 ml) contained: AADC (390 units), 20 mM phosphate buffer pH 7.2, 10 mM mercaptoethanol and DFMD at the concentrations indicated. The mixtures were incubated for 30 min at 37°. Enzyme activities were determined on 40 μl aliquots and the remaining parts were dialysed (at 40°) against 500 ml of 50 mM phosphate buffer containing 10 mM mercaptoethanol and 10 μM pyridoxal phosphate with 2 changes of buffer per 24 hr. Enzyme activity before dialysis was 5190 units per mg of protein in the control and there was no detectable loss of activity after 48 hr dialysis.

Binding of [^{3}H]DFMD to AADC. Binding of [^{3}H]DFMD to AADC was determined as described in Materials and Methods. Saturation of binding of the tritiated inhibitor to the enzyme was obtained in 10 min (Fig. 3). Inhibition of the enzyme proceeded in parallel with binding. It was almost complete after 10 min; 50 per cent of maximum binding and 50 per cent of enzyme inhibition was observed at 1.5–2 min.

When the enzyme was saturated by [^{3}H]DFMD, it was found that 0.83 mole of DFMD was bound to one mole of enzyme. For the calculation, it was assumed that DFMD binds only to the active form of AADC (specific activity 8000 units/mg, mol. wt 112,000 dalton [6]) and not to the degradation product of the enzyme which formed during storage.

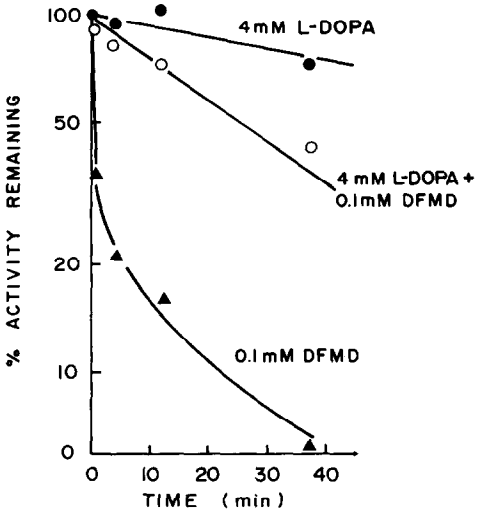

Fig. 2. Protection of AADC by L-DOPA against inhibition by DFMD. Incubation and AADC measurements were done as described in the legend to Fig. 1.

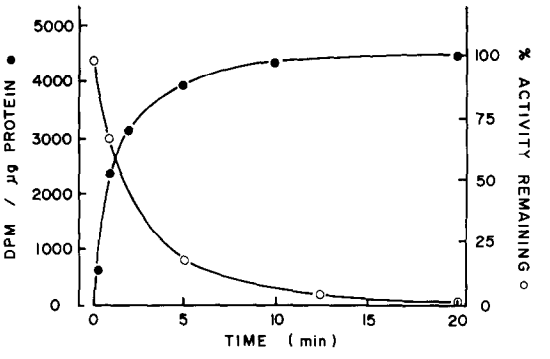

Fig. 3. Correlation between binding of DFMD to AADC and loss of enzyme activity. Purified AADC (3053 units per mg, 0.15 mg/ml) was incubated with 20 μM[^{3}H]DFMD (0.7 Ci/mmole) as indicated in Materials and Methods. At various time intervals, 100 μl aliquots were withdrawn, assayed for remaining enzyme activity (○) and acid-insoluble radioactivity (●).

Effect of DFMD on the absorption spectrum of AADC. The absorption spectrum of purified AADC (0.75 mg/ml), dissolved in 10 mM phosphate buffer containing 5 mM mercaptoethanol, is shown in Fig. 4. It has two bands, one at 420 nm and one at 340 nm. By adding DFMD, there was a modification of the spectrum as shown in Fig. 4. The absorption band at 420 nm decreased slowly with a complete disappearance within 15 min. The absorption band at 340 nm was shifted to 335 nm; at the same time the maximum at 335 nm increased slightly immediately after mixing and then remained constant. This rapid change of the absorption spectrum might be related to the rapid inhibition of the enzyme at high concentrations of DFMD (see Fig. 1).

Metabolism of DFMD in vitro. The flattening of the curves of Fig. 1 suggested that either a substantial part of the inhibitor was removed from the medium or that the enzyme became protected by a reaction product. However, when DFMD was incubated for up to 24 hr with AADC, but in the absence of PLP, no change in its concentration was found and no metabolite was detectable in the incubation medium (Table 2). Under the same experimental conditions, L-DOPA was totally transformed to dopamine (data not shown). In contrast, in the presence of PLP, concentrations of DFMD and PLP in the incubation mixture were decreased in similar amounts. This decrease occurred even when no enzyme was present, suggesting that a non-enzymatic reaction between DFMD and PLP occurred. A new substance was detected in the reaction mixture by h.p.l.c. with a retention time of 25.5 min (DFMD 8.8 min; PLP 6.7 min).

Condensation of DFMD with PLP. Spectra of an initial mixture of equimolar amounts of DFMD and PLP showed a time-dependent decrease of the 390 nm absorption band with a new band appearing around 330 nm, thus indicating a decrease of the amount of free PLP and the formation of a new compound.

The new compound was purified as indicated in Materials and Methods. Its absorption spectrum showed two maxima (325 and 288 nm) (Fig. 5). This spectrum is similar to the spectrum of the tetrahy-

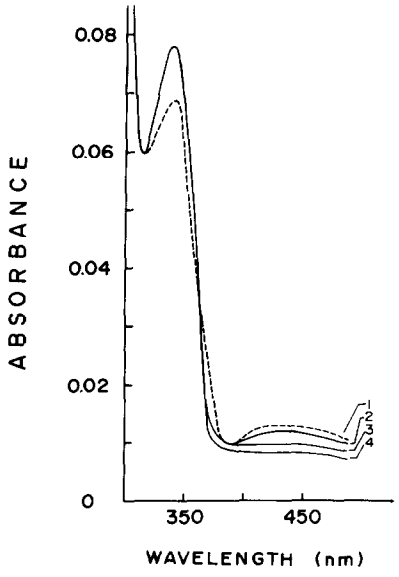

Fig. 4. Effect of DFMD on the absorption spectrum of AADC. Curve 1 : enzyme (0.75 mg/ml, 8009 units/mg) alone; curve 2 : enzyme + 75 μM DFMD immediately after mixing ; curve 3 : enzyme + 75 μM DFMD after 5 min; curve 4 : enzyme + 75 μM DFMD after 15 min.

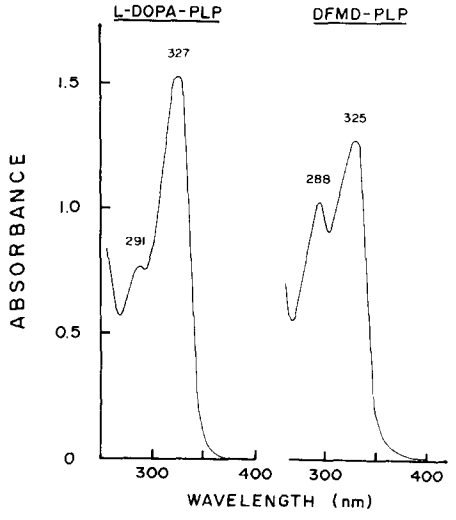

Fig. 5. Absorption spectra of the condensation product of L-DOPA and DFMD with pyridoxal phosphate (PLP). Concentration: 0.2 mM.

droisoquinoline which is formed by the condensation of L-DOPA with PLP (Fig. 5). The elemental analysis and the ^1H nuclear magnetic resonance spectrum (data not shown) of the DFMD–PLP condensation product corresponded to the structure of the tetrahydroisoquinoline shown in Fig. 6.

O-Methylation of DFMD. When [^3H]DFMD was incubated with partially purified catechol-*O*-methyltransferase in the presence of the co-substrate *S*-adenosyl-L-methionine, a radioactive reaction product was detected (Fig. 7). If *S*-adenosyl-L-methionine was omitted from the incubation mixture, DFMD was not transformed. It is therefore suggested that DFMD is a substrate for catechol-*O*-methyltransferase. It was not determined which one of the two possible *O*-methyl derivatives was formed.

Twenty-four hours after oral administration of [^3H]DFMD to rats, two radioactive spots were separated from urine chromatographed on cellulose t.l.c. plates. One had the chromatographic characteristics of DFMD and the other that of the metabolite formed *in vitro* by catechol-*O*-methyltransferase catalysis.

Incubation of urine with glucuronidase and arylsulfatase at 37° for 1 hr did not produce a change in the chromatographic behaviour of the metabolite. Furthermore, when the catechols of the urine were fixed on alumina, then extracted and chromatographed, only one spot corresponding to DFMD was obtained, confirming the non-catechol nature of the metabolite.

DISCUSSION

DFMD inhibits purified hog kidney AADC in a time-dependent manner. The inhibition cannot be reversed by dilution or extensive dialysis. The presence of L-DOPA markedly slows the rate of inhibition. Using ring-tritiated DFMD it was found that

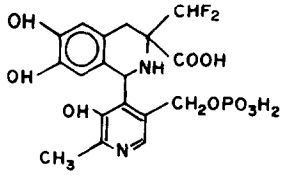

Fig. 6. Structure of the tetrahydroisoquinoline formed by condensation of DFMD with pyridoxal phosphate (PLP).

Table 2. *In vitro* interaction between DFMD and pyridoxal phosphate (PLP)*

Time of incubation (hr)	Control (without enzyme)			AADC added		
	No PLP	+ PLP		No PLP	+ PLP	
	DFMD (μM)	DFMD (μM)	PLP (μM)	DFMD (μM)	DFMD (μM)	PLP (μM)
0	200	200	50	200	200	50
5	184	188	22	190	168	35
24	200	150	8	194	152	10

* The incubation mixtures (1 ml) contained: 50 mM phosphate buffer, pH 7.2, 10 mM mercaptoethanol and, when indicated, 200 μM DFMD, 50 μM PLP and 40 μg of AADC (4640 units per mg of protein). The other conditions were as indicated in Materials and Methods.

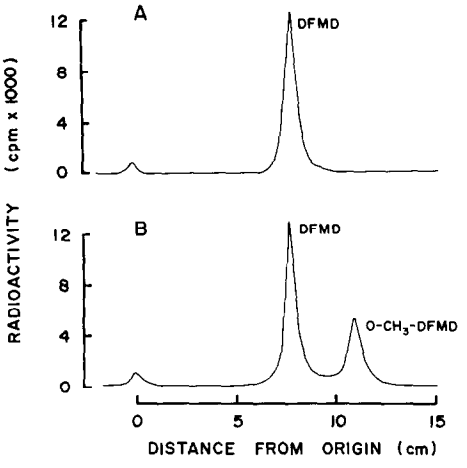

Fig. 7. Reaction of [^{3}H]DFMD with catechol-*O*-methyltransferase. Panel A: radiochromatogram of the incubation mixture at 0 time. Panel B: radioactivity pattern after incubation for 15 min. [Ring-2,5,6-^{3}H]DFMD (100 μM, 100 μCi) was incubated with 70 μM *S*-adenosyl-L-methionine, 125 μM $MgCl_2$ at pH 7.4 in 10 mM phosphate buffer, at 30°. The reaction was stopped by addition of acetic acid and the mixture chromatographed on t.l.c. cellulose plates using butanol–acetic acid–water (60 : 20 : 20) as solvent. Radioactivity was recorded with a radioactivity scanner.

the inhibitor was bound, probably covalently, to the protein and that the enzyme inhibition developed at a rate similar to the incorporation of radioactivity into the protein. The binding was nearly stoichiometric (0.83 mole of inhibitor bound per mole of AADC) indicating that a specific alkylation occurred. Thus, DFMD might be a useful tool to label the active site of the enzyme and to determine its amino acid sequence.

The absorption spectrum of AADC was slightly modified during enzyme inactivation. The observed spectral changes were smaller than those described by Borri-Voltattorni and Minelli for carbidopa (L-α-methyl-α-hydrazino-3.4-dihydroxy-phenyl-propionic acid) [9]. Indeed the hydrazino group of carbidopa binds stoichiometrically to the aldehyde function of the cofactor PLP, and thus induces a profound modification of the spectrum of the enzyme. In contrast, DFMD should mainly bind to a specific amino acid residue of the active site, producing less dramatic spectral changes.

Although in the present study the loss of CO_2 during enzyme inactivation has not been examined, as for the related compound *S*-α-monofluoromethyl DOPA [4], the above data indicate that the inhibition by DFMD is consistent with an enzyme-activated mechanism.

The rate of inhibition by DFMD deviated markedly from pseudo-first-order kinetics; a flattening of the inhibitory curves was observed (Fig. 1). The decrease of the rate of inhibition by DFMD did not correlate with a noticeable disappearance of the compound from the incubation medium and appearance of a reaction product of the inhibitor. As proposed by Maycock *et al.* [10], the existence of isozymes of AADC may explain this phenomenon.

DFMD and PLP were found to react spontaneously to form most likely a tetrahydroisoquinoline (Fig. 6) by the well-known Pictet–Spengler reaction [11]. The purified reaction product formed from DFMD and PLP had no inhibitory effect on AADC and tyrosine hydroxylase activity. This compound was not detected in tissues or urine of DFMD-treated rats under the experimental conditions described in this paper.

DFMD is a substrate of catechol-*O*-methyltransferase *in vitro* and *in vivo*. The presence of *O*-methylated DFMD was demonstrated in urine, liver and kidney of DFMD-treated rats. The use of tritiated DFMD did not allow us to determine the *in vivo* rate of this reaction because a large proportion of the tritium (80 per cent in 4 hr) of [ring-2,5,6-^{3}H]DFMD was exchanged with hydrogen from water. This exchange also took place with ring-triated L-DOPA and indicates that metabolic studies using ring-tritiated L-DOPA should be interpreted with caution.

In conclusion, DFMD is a selective and potent enzyme-activated inhibitor of purified hog kidney AADC. *In vivo*, the major metabolic transformation of DFMD appears to be *O*-methylation.

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