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IN VITRO AND *IN VIVO* EFFECTS OF α-ACETYLENIC DOPA AND α-VINYL DOPA ON AROMATIC L-AMINO ACID DECARBOXYLASE

G. RIBÉREAU-GAYON, C. DANZIN, M. G. PALFREYMAN, M. AUBRY, J. WAGNER, B. W. METCALF and M. J. JUNG

Centre de Recherche Merrell International, 16, rue d'Ankara, 67084-Strasbourg Cedex, France

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Abstract—*In vitro*, α-acetylenic DOPA (RMI 71.858) is a potent inhibitor of aromatic L-amino acid decarboxylase (AADC). Inhibition appears to consist of both a competitive, with a K_i of 0.3 μM, and an irreversible component, the enzyme losing 50 per cent of its activity in 20 min at an inhibitor concentration of 100 μM. After inhibition, the activity can only be partially restored by dialysis. α-Vinyl DOPA (RMI 71.816) is a less potent inhibitor of the enzyme, with a K_i of 39 μM. No transformation of the inhibitors by AADC can be detected in the incubation medium. *Ex vivo*, both compounds (100–500 mg/kg, i.p.) reduce the activity of AADC in different organs, with a more pronounced effect in peripheral tissues than in brain. *In vivo*, α-acetylenic DOPA (10–500 mg/kg, i.p.) inhibits the peripheral decarboxylation of [^{3}H] L-DOPA and 5-hydroxytryptophan with a consequent short-lasting elevation of brain catecholamines and serotonin.

Aromatic L-amino acid decarboxylase (E.C. 4.1.1.26) (AADC) is an enzyme essential for the biosynthesis of a number of biogenic amines in both the peripheral and central nervous systems. The enzyme is not normally rate-limiting but its inhibition can alter the metabolism of exogenously supplied substrates such as L-DOPA or L-5-hydroxytryptophan. Several inhibitors of this enzyme are known, e.g. L-α-methyl-α-hydrazino-3,4-dihydroxy-phenyl-propionic acid (carbidopa) [1] and *N*-(D,L-seryl-*N'*-(2,3,4 trihydroxybenzyl) hydrazine-HCl (benserazide) [2]. These compounds are irreversible inhibitors of AADC. *In vivo*, they show a marked selectivity for the enzyme in peripheral tissues over that of central nervous system. They have been used clinically in combination with L-DOPA for the treatment of Parkinson's disease. Since hydrazine derivatives interact with many pyridoxal phosphate-dependent enzymes, the available inhibitors are not entirely specific for AADC.

Recently the synthesis of two new analogues of L-DOPA: α-acetylenic DOPA (RMI 71.858, α-ethynyl-3-hydroxytyrosine) and α-vinyl DOPA (RMI 71.816, α-vinyl-3-hydroxytyrosine) has been reported [3, 4]. This paper describes the *in vitro* and *in vivo* effects of these compounds on AADC.

MATERIALS AND METHODS

Purification of AADC. The enzyme was purified from hog kidney by an abbreviated modification of the methods of Christenson *et al.* [5] and Lancaster and Sourkes [6], as follows:

(1) *Homogenisation.* Fresh hog kidney cortex (108 g) was homogenised in cold NaCl 0.9% (220 ml) containing 10 mM mercaptoethanol. The homogenate was then centrifuged at 20,000 *g* for 30 min.

(2) *Heat treatment.* To the supernatant was added 2×10^{-4} M pyridoxal phosphate and the solution was heated at 50° for 5 min. It was then cooled in ice and centrifuged at 20,000 *g* for 20 min.

(3) *Ammonium sulfate fractionation.* To the supernatant, a neutralised cold saturated solution of ammonium sulfate was added slowly in order to obtain 32 per cent saturation. After 20 min stirring, the suspension was centrifuged for 20 min at 20,000 *g* and the precipitate was discarded. The supernatant was brought to 49 per cent saturation and after 20 min stirring was centrifuged. The precipitate was dissolved in 50 mM phosphate buffer, pH 7.2, containing 50 mM mercaptoethanol (18 ml). This enzyme solution was then dialysed for 36 hr against 1 liter of the same buffer with one change of buffer.

(4) *DEAE-Sephadex fractionation.* The dialysed solution was loaded on to a 2.5 × 60 cm column of DEAE-Sephadex A-50. The equilibration and development of the column was performed as described by Christenson *et al.* [5]. The fractions containing enzyme activity were concentrated by ultra-filtration and dialysed for 36 hr against 1 liter of 10 mM phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol, with one change of buffer.

(5) *Hydroxylapatite column chromatography.* The hydroxylapatite column (2.5 × 50 cm) was prepared as described by Christenson *et al.* [5]. It was developed with a non-linear phosphate gradient pH 7.2, in the presence of 10 mM mercaptoethanol using an LKB 11300 Ultrograd gradient mixer. Phosphate concentration was increased from 10 to 50 mM during the elution of the first 200 ml. This phosphate concentration was kept constant for a further 130 ml and then increased gradually to 200 mM during the elution of another 400 ml buffer. The flow rate was 8 ml/hr. The fractions containing peak AADC activity were concentrated by ultra-filtration. The specific activity of the enzyme in these fractions (7,500–8,000 units/mg) was in the range of that obtained by Christenson *et al.* [5] for the

homogenous enzyme. The half-life of the enzyme was about 6 weeks at 0°.

AADC activity measurement. Enzyme activity was determined by the CO_2 trapping method described by Christenson *et al.* [5]. One unit of activity is defined as the total amount of enzyme which produces 1 nmole of CO_2 per min.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* [7].

In vitro *metabolism of α-acetylenic DOPA.* α-Acetylenic DOPA was measured in incubation mixtures by high performance liquid chromatography (HPLC) as follows: to 850 μl of 50 mM phosphate buffer pH 7.2 containing 10 mM mercaptoethanol, 100 μl of 2 mM α-acetylenic DOPA and 50 μl of enzyme (40 μg protein) were added. After various times of incubation at 20°, 100 μl aliquots were mixed with 20 μl of 0.6 M trichloroacetic acid. Control incubations were performed without enzyme. Aliquots of the trichloroacetic acid solution (10 μl) were directly applied on a 30 cm × 3.5 mm μBondapak C_{18} column. The two following eluants were used: (1) acetic acid 0.02 M, (2) citric acid, 0.1 M (795 ml) mixed with Na_2HPO_4, 0.2 M (205 ml). The flow rate was 1 ml/min. A Waters model 204 liquid chromatograph, consisting of a 6000A solvent delivery system, a UGK universal injector and a u.v. absorbance detector, was employed. The absorbance of the eluant was monitored at 280 nm.

Ex vivo *AADC activity of rat brain, heart and kidney.* Male rats (Sprague–Dawley 200–250 g, from Charles River, France) were dosed i.p. with 100 or 500 mg/kg α-acetylenic DOPA or α-vinyl DOPA or with saline. Four hours later they were sacrificed and the AADC activity of brain, heart and kidney was measured. The procedure involved homogenisation of tissue in nine vol. 50 mM phosphate buffer, pH 7.0, and incubation of 500 μl (brain or heart) or 50 μl (kidney) of the homogenate with 0.3 mM D,L-DOPA-1-[^{14}C] (0.1 μCi) in a final volume of 1 ml completed with homogenisation buffer, at 37° for 15 min. AADC activity was determined by the CO_2 trapping method [5].

In vivo *metabolism of* [^{3}H]-L-*DOPA.* Groups of 5 male mice (25–30 g CD_1 albino, Charles River, France) were injected i.p. with various doses of α-acetylenic DOPA (10–500 mg/kg) and 1 mCi/kg [^{3}H]-L-DOPA (L-3,4-dihydroxy [ring 2,5,6-^{3}H] phenylalanine, 30 Ci/m-mole, Amersham). Half an hour later the mice were decapitated and the brains homogenised in 4 ml 0.4 N perchloric acid containing 0.1% (w/v) EDTA and 0.05% (w/v) sodium metabisulphite. [^{3}H] L-DOPA, [^{3}H]noradrenaline and [^{3}H]dopamine were separated on Dowex 50W-X4 ion exchange columns by the method of Claxton *et al.* [8].

In vivo *metabolism of 5-hydroxytryptophan.* Groups of 6 mice were injected i.p. with 100 mg/kg α-acetylenic DOPA and 150 mg/kg i.p. of D,L-5HTP, or with 150 mg/kg i.p. D,L-5HTP alone. At various times after injection the mice were sacrificed by decapitation and the serotonin content of the brains and hearts measured by the fluorometric method of Snyder *et al.* [9].

Chemicals. α-Acetylenic DOPA and α-vinyl DOPA were synthesized by Metcalf and Jund [4]. All other compounds were of analytical grade and commercially available.

RESULTS

Irreversible inhibition of AADC by α-acetylenic DOPA and α-vinyl DOPA. Incubation of AADC with α-acetylenic DOPA results in a time-dependent loss of enzyme activity, as shown in Fig. 1. After a rapid initial drop of activity, the inhibition develops more slowly. Even at 200 μM α-acetylenic DOPA, approximately 10 per cent of enzyme activity remains after 1 hr of incubation. At 100 μM α-acetylenic DOPA the enzyme loses 50 per cent of its activity in about 20 min. For α-vinyl DOPA the pattern of inhibition is similar but the flattening of the curves is even more pronounced.

The flattening of the curves suggested that a substantial part of the inhibitors was removed from the medium or that the enzyme became protected by a reaction product. However, when the concentration of α-acetylenic DOPA was measured by HPLC after its incuba-

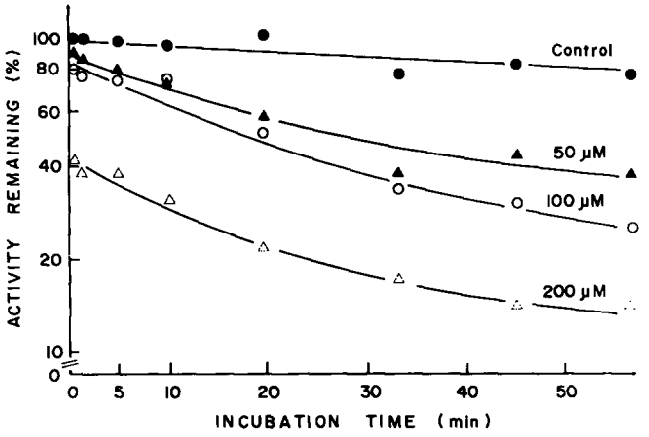

Fig. 1. Time-dependent inhibition of hog kidney aromatic amino acid decarboxylase (AADC) by α-acetylenic DOPA. To 100 μl of 50 mM phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol were added 50 μl of an aqueous solution of the inhibitor and 50 μl of enzyme solution (40 μg protein). This mixture was incubated at 37° and 20 μl aliquots were withdrawn to measure AADC activity at the times indicated.

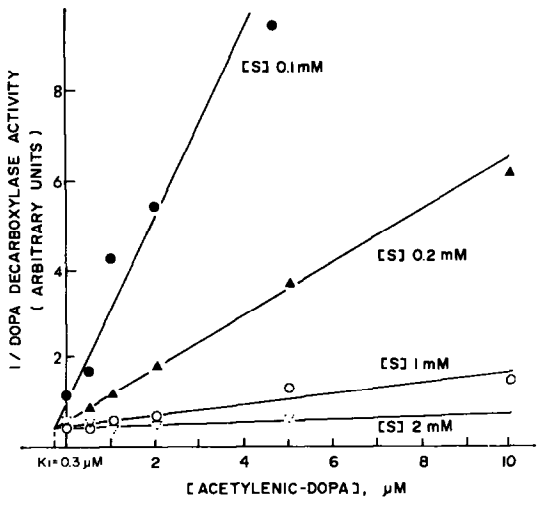

Fig. 2. Competitive inhibition of aromatic amino acid decarboxylase by α-acetylenic DOPA. The substrate, L-DOPA (S), had for each determination the same specific radioactivity: 15.400 c.p.m./μmole. A mixture of inhibitor and substrate in a total volume of 0.5 ml was prepared for each enzyme activity determination and added to the incubate after activation of the enzyme by pyridoxal phosphate, as described by Christenson *et al.* [5].

tion with enzyme, no change in its concentration was detected over a period of 24 hr incubation, and no metabolite of α-acetylenic DOPA was detectable in the incubation medium. Under the same incubation conditions, DOPA was totally transformed to dopamine. The precision of the technique allowed us to detect the transformation of 5 per cent of the α-acetylenic DOPA.

In order to determine if the observed inhibition was irreversible, the enzyme, incubated for 30 min with α-acetylenic DOPA, at different concentrations, was extensively dialysed (at 4°) against 50 mM phosphate buffer containing 10 mM mercaptoethanol and 10^{-5} M pyridoxal phosphate. This buffer was changed twice a day. With 200 μM α-acetylenic DOPA, 22 per cent of the enzyme activity was recovered after 24 hr of dialysis and with 20 μM α-acetylenic DOPA, 34 per cent was recovered after 24 hr. The data obtained after 48 and 72 hr of dialysis were not interpretable because of the instability of the enzyme under these conditions.

Determination of Michaelis–Menten constants. Because of the non-pseudo-first-order nature of the irreversible inhibition, the K_i's of α-acetylenic DOPA and α-vinyl DOPA were determined in a classical manner using conditions where little irreversible inhibition occurred (Fig. 3). α-Acetylenic DOPA appeared to inhibit competitively AADC with a K_i of 0.3 μM (Fig. 2); α-vinyl DOPA was less potent with a K_i of 39 μM (Fig. 3). The K_m for L-DOPA was 140 μM, in agreement with the value reported by Christenson *et al.* [5].

Effect of α-acetylenic DOPA and α-vinyl DOPA on AADC activity of three tissues of the rat. From Table 1 it can be seen that α-acetylenic DOPA and α-vinyl DOPA produce a dose-related inhibition of the activity of AADC in brain, heart and kidney of the rat. At a dose of 500 mg/kg i.p. some inhibition of the enzyme occurs in the brain, but with both compounds the degree of inhibition is higher in the periphery than in the brain.

Effect of α-acetylenic DOPA on [^{3}H]DOPA metabolism in vivo. As can be seen from Fig. 4, α acetylenic DOPA produces a dose-related increase in the concentrations of [^{3}H]DOPA and [^{3}H]catecholamines in the brains of mice after i.p. doses of 10–500 mg/kg.

Effect of α-acetylenic DOPA on 5HTP metabolism in vivo. When 100 mg/kg α-acetylenic DOPA is co-administered i.p. with 150 mg/kg 5HTP, it significantly increases the concentrations of serotonin in the brain of mice as compared to 5HTP alone (Fig. 5A). This increased serotonin concentration is still evident 8 hr after dosing. In the same mice, co-administration of α-acetylenic DOPA with 5HTP (Fig. 5B) markedly reduces the accumulation of serotonin in the hearts when compared with 5HTP administration alone. However, the level of serotonin in the heart is no longer different from control values 2 hr after dosing.

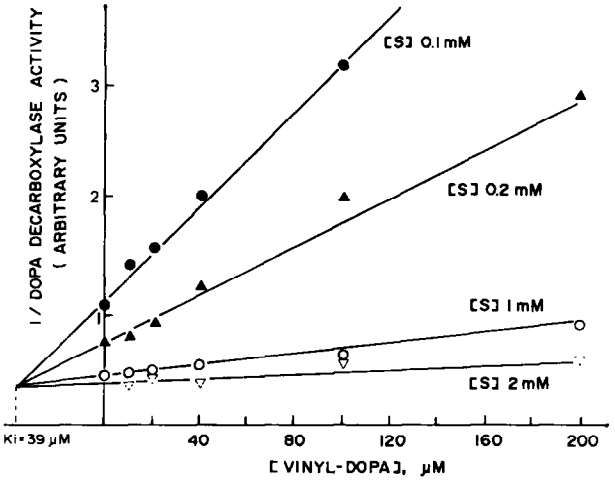

Fig. 3. Competitive inhibition of aromatic amino acid decarboxylase by α-vinyl DOPA. Conditions as described in Fig. 2.

Table 1. The effect of α-acetylenic DOPA and α-vinyl DOPA on aromatic amino acid decarboxylase activity of three tissues of the rat 4 hr post dose

	Remaining activity (% control)		
	Brain	Heart	Kidney
Saline*	100 ± 4	100 ± 10	100 ± 19
α-Acetylenic DOPA, 100 mg/kg, i.p.	99 ± 1	82 ± 5†	50 ± 4†
α-Acetylenic DOPA, 500 mg/kg, i.p.	80 ± 4‡	59 ± 5‡	41 ± 4‡
α-Vinyl DOPA, 100 mg/kg, i.p.	90 ± 2	75 ± 7	60 ± 8
α-Vinyl DOPA, 500 mg/kg, i.p.	85 ± 3†	60 ± 15	36 ± 3‡

* Control values in nmoles/min/g (mean + S.E.M.) were 55 ± 2 for brain, 64 ± 6 for heart and 1144 ± 220 for kidney.

$n = 5$.

† $P < 0.05$ as determined by Student's t test.

‡ $P < 0.005$ as determined by Student's t test.

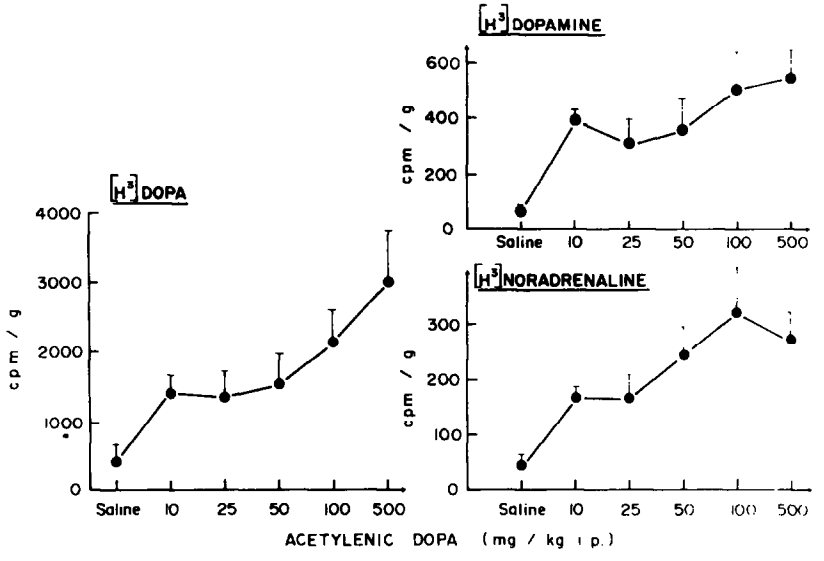

Fig. 4. Effect of pretreatment with α-acetylenic DOPA on brain levels of [^{3}H]DOPA, [^{3}H]dopamine and [^{3}H]noradrenaline. Groups of five mice were injected i.p. with various doses of α-acetylenic DOPA (10–500 mg/kg) and with 1 mCi/kg i.p. [^{3}H]-L-DOPA. Mice were sacrificed 30 min later. A further group of 5 mice injected with saline and [^{3}H]-L-DOPA served as controls. The graphs show the [^{3}H]DOPA, [^{3}H]dopamine and [^{3}H]noradrenaline content of the brain (c.p.m./g, mean ± S.E.M.). All values are statistically significantly ($P < 0.05$) greater than saline controls (Student's t test, 2 tailed).

DISCUSSION

α-Acetylenic DOPA and α-vinyl DOPA were synthesized as potential enzyme-activated irreversible inhibitors of AADC [4]. It is evident from the above results that these compounds are only weakly effective in this respect. Dialysis for 24 hr of the inhibited enzyme partially restored its activity thus indicating that the enzyme-α-acetylenic DOPA complex is slowly dissociable.

α-Acetylenic DOPA and α-vinyl DOPA have a remarkable competitive component in their inhibitory action with a K_i of 0.3 μM and 39 μM respectively. For comparison, α-methyl DOPA, a known competitive inhibitor of AADC, has a K_i of around 50 μM as reported by Lovenberg *et al.* [10]. The competitive inhibition may be the cause for the low initial enzyme activity (Fig. 1). This is in agreement with the data of Aster *et al.* [11].

During the inhibition process no detectable decrease of drug concentration was observed in the incubation medium and no reaction product was found. Thus, there is presently no clear explanation for the flattening of the curves of Fig. 1.

α-Acetylenic DOPA and α-vinyl DOPA are equipotent inhibitors of AADC in various tissues when administered to rats. At a large dose (500 mg/kg i.p.) there is evidence for a 15–20 per cent inhibition of the brain enzyme. The same dose inhibits the enzyme in the kidney by approximately 60 per cent. Thus, there is some selectivity towards the enzyme in peripheral tissues. It seemed likely that co-administration of L-DOPA or 5HTP with α-acetylenic DOPA would allow

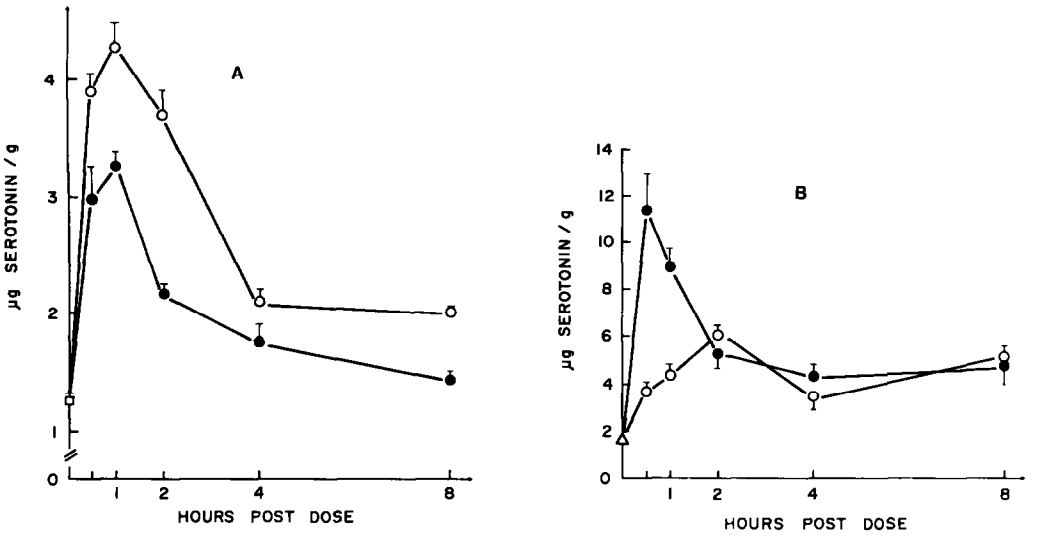

Fig. 5. Effect of pretreatment with α-acetylenic DOPA on serotonin formation from 5-hydroxytryptophan in brain (A) and heart (B). Groups of 5 mice were injected i.p. with 100 mg/kg α-acetylenic DOPA plus 150 mg/kg 5HTP (○—○), with 150 mg/kg 5HTP alone (●—●) or with saline (and △). The animals were sacrificed at various times after injection of α-acetylenic DOPA. Saline-injected animals served as zero time controls. Serotonin concentrations (μg/g, mean + S.E.M.) are shown in brain (A) and heart (B).

more L-DOPA or 5HTP to penetrate into the brain over that found in animals not treated with the inhibitor. Subsequent decarboxylation should increase the concentration of brain dopamine and serotonin respectively. That this assumption was correct is shown by the dose-related increase in [^{2}H]DOPA and [^{3}H]catecholamines observed in the brains of mice following peripheral co-administration of α-acetylenic DOPA and [^{4}H]-L-DOPA and the even greater increase in brain serotonin following α-acetylenic DOPA plus 5HTP when compared to 5HTP alone. In addition, the increase in heart serotonin following 5HTP was greatly attenuated when 5HTP was administered together with α-acetylenic DOPA. However, the inhibition of AADC in peripheral tissues of mice is of short duration, e.g. less than 2 hr in the heart.

Although it appears that α-acetylenic DOPA inhibits AADC in the heart of mice for less than 2 hr, there is still 18 per cent inhibition of the enzyme in the heart of rats 4 hr after dosing. It is likely that this degree of inhibition is insufficient to modify the decarboxylation of either L-DOPA or 5HTP. It is known that AADC is present in large excess of requirements in many tissues and substantial inhibition of the enzyme is required to modify the metabolism of 5HTP or L-DOPA [12].

Carbidopa and benserazide inhibit AADC at lower concentrations than does either α-acetylenic DOPA or α-vinyl DOPA, presumably due to the high affinity of the hydrazine group for AADC bound pyridoxal phosphate [2]. For instance 10^{-8} M carbidopa was found to inhibit purified AADC by 50 per cent in the presence of 1 mM L-DOPA. α-Acetylenic DOPA and α-vinyl DOPA, which belong to a different class of compounds, have a different mechanism of action. α-Difluoromethyl DOPA, another enzyme-activated irreversible inhibitor of AADC, has recently been reported [12]. This compound is a more potent irreversible inhibitor than α-acetylenic DOPA and α-vinyl DOPA, the half-life of purified AADC being 1 min at 100 μM. However, the product of reaction between enzyme and α-difluoromethyl DOPA is not dissociable [12], in contrast with the corresponding complexes with α-acetylenic DOPA and α-vinyl DOPA. The nature of the bonds between the enzyme and the different inhibitors should therefore be different. These interactions are currently being investigated.

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