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INHIBITION OF TYROSINE HYDROXYLASE *IN VITRO* AND *IN VIVO* BY 3-AMINO-PYRROLO[3,4c]ISOXAZOLE AND DERIVATIVES

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Abstract—Tyrosine hydroxylase was shown to be inhibited *in vitro* by a series of 3-amino-pyrrolo[3,4c]isoxazoles. Greatest inhibition was observed with ethyl 3-amino-4H-pyrrolo[3,4c]isoxazole-5(6H) carboxylate and other 5-carboxylates. Acylation of the 3-amine group greatly decreased inhibition. Inhibition by ethyl 3-amino-4H-pyrrolo-[3,4c]isoxazole carboxylate was noncompetitive with tyrosine or pteridine cofactor, but could be reversed by addition of iron or copper. 3-Amino-pyrroloisoxazoles were found to form metal complexes with Fe^{2+}, suggesting that their inhibition may be due to chelation. Tyrosine hydroxylase activity of adrenal extracts was decreased in rats treated with ethyl-3-amino-4H-pyrrolo[3,4c]isoxazole carboxylate and its *N*-acetylated derivative.

BRAIN, adrenal medulla, and several sympathetically innervated tissues contain an L-tyrosine hydroxylase which catalyzes the initial and rate-limiting step in the biosynthesis of norepinephrine.[1, 2] The enzyme has been partially purified from bovine adrenal medulla[1] and shown to require a structurally specific pteridine cofactor.[3, 4] Three classes of compounds have been reported to inhibit tyrosine hydroxylase *in vitro*. Certain aromatic amino acids inhibit the enzyme by competing with the tyrosine substrate.[5] A large number of catechols as well as a catechol isostere, 4-isopropyltropolone, also inhibit the enzyme by competing with the pteridine cofactor.[5–7] In a preliminary communication, we reported the inhibition *in vitro* of tyrosine hydroxylase by a third class of compounds, pyrroloisoxazoles.[8] The present communication reports on the mechanism of tyrosine hydroxylase inhibition by pyrroloisoxazoles *in vitro* and gives evidence for their direct inhibition of adrenal tyrosine hydroxylase activity *in vivo*.

EXPERIMENTAL

Materials. The syntheses of the novel pyrroloisoxazoles used in this study have been described elsewhere.[9] The compounds are relatively insoluble in water, and were dissolved in dimethylformamide or in 10% aqueous ethanol for use in these studies. α-Methyl-*p*-tyrosine was obtained from Merck, Sharp & Dohme Research Laboratory; 3,4-dihydroxyphenylpropylacetamide (H-22/54) from Hässle Laboratories, Sweden; and *p*-bromo-*m*-hydroxybenzyloxyamine (NSD-1055) from Smith and Nephew Research, Ltd.

Assay in vitro. Tyrosine hydroxylase was prepared from beef adrenal medulla according to the procedure of Nagatsu *et al.*[1] The 0–40% ammonium sulfate precipitate

was resuspended in 1×10^{-3} M aqueous mercaptoethanol (pH 6·5) and stored at −20°. Tyrosine hydroxylase activity was assayed by measuring the tritiated water formed from L-tyrosine-3,5-^{3}H as reported by Nagatsu *et al.*[10] The standard incubation mixture contained: 200 μmole acetate buffer (pH 6·0); 0·1 μmole L-tyrosine containing $2·2 \times 10^4$ cpm of L-tyrosine-3,5-^{3}H; 1·0 μmole 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) added in 0·1 ml of 0·1 M phosphate (pH 7·4) containing 1 M mercaptoethanol; 0·1 μmole NSD-1055; and 0·25 ml of enzyme preparation. The pyrroloisoxazoles were added in 0·1 ml of solvent. The final volume was adjusted to 1·0 ml with water. Incubations were for 30 min at 37° in a metabolic shaker. The reaction was stopped by the addition of 0·05 ml glacial acetic acid and the mixture was centrifuged. The supernatant solution was placed on a Dowex-50(H^+) column[4] and washed with 1·0 ml water. The extract and washings were mixed with 10 ml of Brays solution, and radioactivity was determined by using a Packard Tri-Carb liquid scintillation counter.

Unless otherwise specified, the enzyme was added last and was not preincubated with the inhibitors or the other reactants. The velocity was found to be linear during the 30-min incubation period at the enzyme concentration used. The inhibition due to the solvent was corrected for by use of controls in each incubation. When ferrous ions were added to the incubations, freshly prepared aqueous solutions of ferrous ammonium sulfate were used. Aqueous solutions of metal chlorides were the source of the other metal ions.

Assay in vivo. The estimation of tyrosine hydroxylase inhibition *in vivo* has previously been made by measurement of tissue catecholamine levels,[11] by determining the conversion of ^{14}C-tyrosine to ^{14}C-norepinephrine,[11] and by measuring tyrosine hydroxylase activity in the press juice of hearts from guinea pigs treated with inhibitors.[6] The latter method has been further developed in our laboratory for estimating inhibition *in vivo* of tyrosine hydroxylase in rat adrenals as described below.

The compounds to be tested were dissolved in an appropriate solvent and injected i.p. into male Sprague–Dawley rats (Carworth Farms, 150–250 g) which had been fasted for 18 hr.

After 3 hr the animals were decapitated and the adrenals were removed, freed of connective tissue and weighed. A 20% (w/v) homogenate of the adrenals of each rat was prepared in 0·32 M sucrose by using a small motor-driven glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min at 0°. An 0·02-ml aliquot of the supernatant fluid was used for assay of tyrosine hydroxylase activity. The remaining incubation mixture consisted of 40 μmole phosphate buffer (pH 6·0), 40 μmole mercaptoethanol, 0·1 μmole $DMPH_4$, and carrier-free L-tyrosine-3,5-^{3}H ($2·2 \times 10^4$ cpm) in a volume of 0·05 ml. The dilution of substrate and inhibitor in the supernatant fluid was less than 20-fold. The total mixture of 0·07 ml was incubated for 20 min at 37° in a metabolic shaker and the reaction was stopped by adding 0·4 ml of 5% (w/v) trichloroacetic acid. After centrifugation, the tritiated water formed was assayed as described above. Acid-containing blanks were used to correct for nonenzymatic hydroxylation.

RESULTS

Comparison with other tyrosine hydroxylase inhibitors. The two pyrroloisoxazoles found to be the most potent inhibitors of tyrosine hydroxylase *in vitro* were ethyl

3-amino-4H-pyrrolo[3,4c]-isoxazole-5(6H) carboxylate (CL-65263) and the corresponding isobutyl ester (Table 1). These compounds were equal in potency to 3,4-dihydroxyphenylpropylacetamide (H-22/54). In our assay system, pyrocatechol and L-norepinephrine inhibited 50 per cent at 5×10^{-3} and 3×10^{-3} M respectively, and *n*-propylgallate at 1×10^{-5} M. The tyrosine analogs, α-methyl-*p*-tyrosine and 3-iodo-L-tyrosine inhibited at $6{\cdot}5 \times 10^{-5}$ and 2×10^{-6} M respectively.

TABLE 1. COMPARISON OF PYRROLOISOXAZOLES WITH OTHER TYROSINE HYDROXYLASE INHIBITORS

Compound	Concn for 50% inhibition * (M)
3-Amino-4H-pyrrolo[3,4c]isoxazole-5(6H)-carboxylic acid, ethyl ester (CL-65263)	1×10^{-4}
3-Amino-4H-pyrrolo[3,4c]isoxazole-5(6H)-carboxylic acid, isobutyl ester	$1{\cdot}2 \times 10^{-4}$
Pyrocatechol	5×10^{-3}
L-Norepinephrine	3×10^{-3}
3,4-Dihydroxyphenylpropylacetamide (H-22/54)	1×10^{-4}
N-propylgallate	1×10^{-5}
DL-α-Methyl-*p*-tyrosine	$6{\cdot}5 \times 10^{-5}$
3-Iodo-L-tyrosine	2×10^{-6}

* Data represent a range of 45–55 % inhibition.

Structure–activity studies. Thirteen pyrroloisoxazole derivatives were compared for their relative inhibition of tyrosine hydroxylase *in vitro* (Table 2). The compounds were compared to CL-65263, the ethyl ester of 3-amino-4H-pyrrolo-[3,4c]isoxazole-5-(6H)-carboxylic acid, which gave the greatest inhibition. The isobutyl ester and benzyl ester were 83 and 25 per cent as potent as the ethyl ester respectively.

Alteration of the amine group of CL-65263 greatly reduced *in vitro* inhibition. The formyl, acetyl and propionyl derivatives were less than 5 per cent as potent as the parent compound.

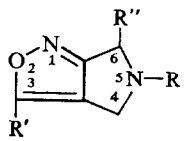
The presence of a methyl group at position-6 also decreased inhibition. The 6-methyl derivative of CL-65263 was only 20 per cent as inhibitory as its parent compound. Acetylation of the 3-amino group of the 6-methyl derivative further reduced potency.

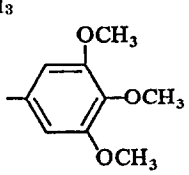
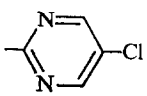
Studies on the mechanism of inhibition in vitro. Ethyl 3-amino-4H-pyrrolo[3,4c]-isoxazole-5(6H)-carboxylate (CL 65263) was selected as representative of the pyrroloisoxazoles for studies on the mechanism of inhibition *in vitro*.

Double reciprocal plots of tyrosine concentration vs. the rate of tyrosine hydroxylation indicated that the inhibition by CL-65263 was noncompetitive with tyrosine over a series of tyrosine concentrations from 4×10^{-6} to 1×10^{-4} M (Fig. 1). These concentrations were in the range of the K_m for tyrosine, which was found to be 5×10^{-5} M. CL-65263 was found to be noncompetitive with the $DMPH_4$ cofactor (Fig. 1) over a range of $DMPH_4$ concentrations from 5×10^{-4} to 5×10^{-3} M.

In a preliminary communication,[8] we reported that the inhibition by CL-65263 was dependent on the concentration of Fe^{2+} in the incubation mixture. This effect is

TABLE 2. EFFECT OF VARIOUS SUBSTITUENTS ON INHIBITORY ACTIVITY OF PYRROLOISOXAZOLES



R	R′	R″	Concn for 50% inhibition (M)	Relative inhibition
—$COOC_2H_5$	—NH_2	H	$1{\cdot}0 \times 10^{-4}$	100
—$COOC_4H_9$ (iso)	—NH_2	H	$1{\cdot}2 \times 10^{-4}$	83
—$COOH_2$— 	—NH_2	H	$4{\cdot}0 \times 10^{-4}$	25
—$COCH_3$	—NH_2	H	$8{\cdot}0 \times 10^{-4}$	12
—CO 	—NH_2	H	$4{\cdot}0 \times 10^{-4}$	25
	—NH_2	H	$1{\cdot}2 \times 10^{-2}$	8
—$COOC_2H_5$	—NHCHO	H	$2{\cdot}0 \times 10^{-3}$	5
—$COOC_2H_5$	—$NHCOCH_3$	H	$1{\cdot}5 \times 10^{-2}$	< 1
—$COOC_2H_5$	—$NHCOC_2H_5$	H	$7{\cdot}0 \times 10^{-3}$	1
—$COOC_2H_5$	—$N{=}C(CH_3)NH_2$	H	$5{\cdot}0 \times 10^{-3}$	2
—$COOC_2H_5$	—$N{=}C(CH_3)NHCH_3$	H	$3{\cdot}0 \times 10^{-3}$	3
—$COOC_2H_5$	—NH_2	CH_3	$5{\cdot}0 \times 10^{-4}$	20
—$COOC_2H_5$	—$NHCOCH_3$	CH_3	$5{\cdot}0 \times 10^{-3}$	2

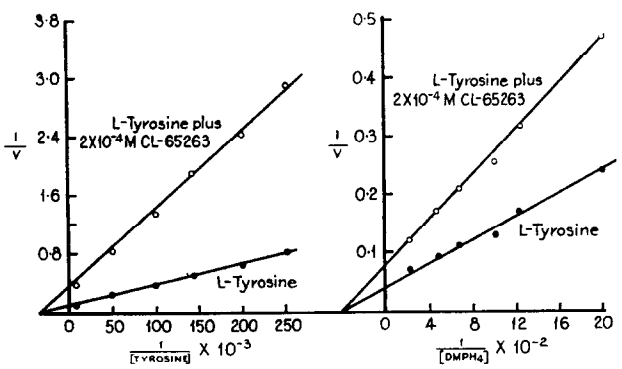


FIG. 1. Double reciprocal plots of tyrosine concentration or $DMPH_4$ concentration vs. rate of tyrosine hydroxylation with and without 2×10^{-4} M CL-65263. At varied tyrosine concentrations, $DMPH_4$ was 1×10^{-3} M; at varied $DMPH_4$ concentrations, tyrosine was 1×10^{-4} M.

shown in Fig. 2. At a concentration of 2×10^{-4} M, CL-65263 inhibited 63 per cent of the unfortified enzyme. The presence of Fe^{2+} in the reaction mixture prevented the inhibition. A similar effect was seen with 2×10^{-5} M *o*-phenanthroline, but less Fe^{2+} was required to prevent inhibition. The inhibition produced by α-methyl-*p*-tyrosine and H-22/54 was unaffected by the presence of Fe^{2+}. When Fe^{2+} was added during the

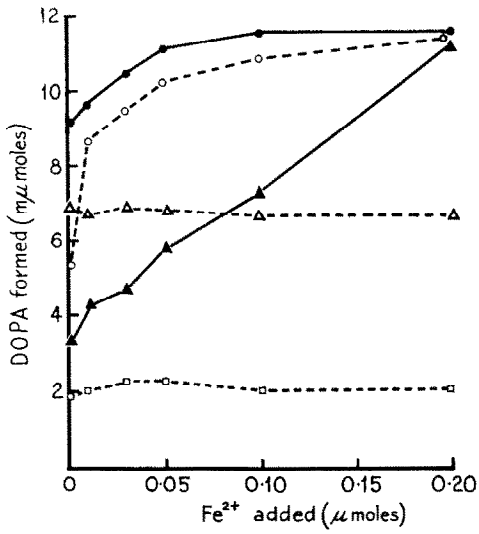


FIG. 2. Effect of ferrous ions on the inhibition of tyrosine hydroxylase by 2×10^{-4} M CL-65263 (▲———▲), 2×10^{-5} M *o*-phenanthroline (○ - - - ○), 2×10^{-5} M α-methyl-*p*-tyrosine (Δ - - - Δ), 2×10^{-4} M H-22/54 (⊡ - - - ⊡) and no inhibitor (●———●).

incubation, a reversal of inhibition was observed (Table 3). In this experiment the inhibition by 2×10^{-4} M CL-65263 was measured at various times during the 30-min incubation period. When Fe^{2+} was added after 10 min of incubation, the inhibition was significantly decreased; when no Fe^{2+} was added, the inhibition remained essentially constant.

TABLE 3. REVERSAL OF INHIBITION BY ADDITION OF FERROUS ION DURING INCUBATION

Incubation time (min)	Per cent inhibition by CL-65263 (2×10^{-4} M)	
	No added Fe^{2+}	Fe^{2+} added (10^{-3} M) at 10 min
10	80	80
15	85	77
20	84	68
25	84	62
30	83	57

Studies were also made to determine whether other metal ions would prevent the inhibition by CL-65263. The metal ions were added to the incubation mixture at 1×10^{-3} M in the presence of 2×10^{-4} M CL-65263. This concentration of CL-65263

gave almost complete inhibition (90 per cent) in the absence of added metal ions. As shown in Table 4, the inhibition by CL-65263 was prevented only by iron and copper ions. Because of the high concentration of mercaptoethanol in the reaction mixture, the ferric ions are probably reduced to ferrous ions.

TABLE 4. PREVENTION OF CL-65263 INHIBITION BY METAL IONS

Metal ion (1×10^{-3} M)	% Inhibition* by CL-65263 (2×10^{-4} M)
None	90 (± 10)
Fe^{2+}	0
Fe^{3+}	8
Cu^{1+}	9
Cu^{2+}	9
Ba^{2+}	88
Ca^{2+}	93
Co^{2+}	83
Hg^{2+}	99
Mg^{2+}	87
Mn^{2+}	91
Ni^{2+}	98
Pb^{2+}	100
Sn^{2+}	100
Sn^{4+}	100
Zn^{2+}	100

* Relative to enzyme activity of respective metal-containing control with no CL-65263.

The inhibition by CL-65263 was found to be pH dependent. At pH values lower than 6·0, the inhibition by CL-65263 was greater than that above pH 6·0 (Table 5). The noninhibited enzyme has a very narrow pH range with maximum activity at pH 6·0.

TABLE 5. EFFECT OF pH ON INHIBITION BY CL-65263

pH	Dopa formed (mμmoles)		Inhibition (%)
	No CL-65263	10^{-4} M CL-65263	
4·8	2·5	0·0	100
5·4	5·6	0·2	97
5·6	6·7	0·8	88
5·8	7·3	1·9	74
6·0	7·8	3·3	58
6·2	6·5	3·2	51
6·6	1·7	1·3	24

Studies in vivo. CL-65263 and its *N*-acetylated derivative were assayed for inhibition *in vivo* by administration of the compounds to rats and assaying the tyrosine hydroxylase activity in extracts of the isolated adrenals, as described above. α-Methyl-*p*-tyrosine was assayed in the same manner. Dose-dependent inhibitions of the adrenal activity were observed for all three compounds (Table 6). α-Methyl-*p*-tyrosine inhibited 52 per cent at a dose of 50 mg/kg. CL-65263 inhibited 45 per cent at 100 mg/kg and its 3-acetylated derivative inhibited 30 per cent at 100 mg/kg.

The ability of α-methyl-*p*-tyrosine and CL-65263 to inhibit the untreated rat adrenal enzyme preparation was tested by adding CL-65263 and α-methyl-*p*-tyrosine to the extracts. At 10^{-4} M, CL-65263 inhibited 51 per cent and α-methyl-*p*-tyrosine inhibited 91 per cent of the tyrosine hydroxylase activity.

TABLE 6. TYROSINE HYDROXYLASE ACTIVITY IN RAT ADRENAL EXTRACTS AFTER ADMINISTRATION OF PYRROLOISOXAZOLES OR α-METHYL-*p*-TYROSINE

	Dose (mg/kg)	cpm*	Inhibition (%)
3-Amino-4H-pyrrolo[3,4c]isoxazole -5(6H)carboxylic acid, ethyl ester (CL-65263)	100	900 ± 69	45
	50	1290 ± 168	22
	0	1648 ± 83	
3-Acetamido-4H-pyrrolo[3,4c]isoxazole -5(6H)carboxylic acid, ethyl ester	100	1060 ± 88	30
	50	1528 ± 65	0
	0	1514 ± 53	
α-Methyl-*p*-tyrosine	250	229 ± 37	88
	100	576 ± 34	69
	50	897 ± 88	52
	0	1880 ± 138	

* Tyrosine hydroxylase activity was measured in adrenal extracts 3 hr after i.p. administration of the compounds. Data represent average from 5 rats ± S.E.

Interaction of pyrroloisoxazoles with metal ions. In preliminary spectral studies, it was observed that solutions of CL-65263 in 0·1 N HCl formed a deep red color in the presence of ferrous ions. This color showed an absorption maximum at 510 mμ. No color was observed with either CL-65263 or ferrous ions alone. Visible spectra were

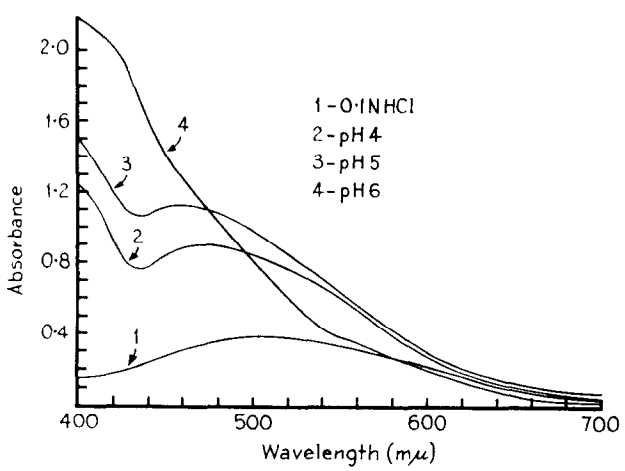


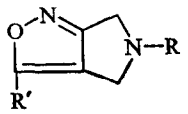
FIG. 3. Absorption spectra of mixtures of CL-65263 and Fe^{2+} as a function of pH.

determined after 30 min on mixtures of CL-65263 (1·25 mg/ml) and ferrous ammonium sulfate (1·25 mg/ml) in 0·05 M acetate buffers at pH 4, 5 and 6 (Fig. 3) with a Cary recording spectrophotometer. Analyses of the spectra revealed the gradual development of an absorption maximum at 470 mμ on lowering the pH from 6 to 4. This

absorption maximum shifted to 510 mμ in 0·1 N HCl. At pH 7 and pH 8, the ferrous ions were rapidly oxidized and formed a precipitate. At similar concentrations of CL-65263 and cupric chloride, no color change was observed, nor was color formation observed with the other metals listed in Table 4.

In an attempt to correlate the color formation at 510 mμ with inhibition *in vitro* of tyrosine hydroxylase, the molar extinction coefficients of several pyrroloisoxazoles at 510 mμ in 0·1 N HCl with Fe^{2+} were determined (Table 7). The most potent enzyme

TABLE 7. EFFECT OF VARIOUS SUBTITUENTS ON FORMATION OF 510 mμ ABSORBING COMPLEX WITH FERROUS ION



R	R′	Molar extinction coefficient (510 mμ)
—$COOC_2H_5$	—NH_2	70·6
—$COOH_2$— 	—NH_2	49·8
—$COCH_3$	—NH_2	45·5
—$COOC_2H_5$	—NHCHO	3·1
—$COOC_2H_5$	—$NHCOCH_3$	1·9
—$COOC_2H_5$	—$N{=}C(CH_3)NH_2$	4·9

inhibitors from Table 2 were found to have the highest molar extinction coefficients at 510 mμ.

DISCUSSION

Since tyrosine hydroxylase appears to be the rate-limiting enzyme in catecholamine synthesis, inhibitors of the enzyme may be of pharmacological value. The tyrosine analogs, α-methyl-*p*-tyrosine and 3-iodotyrosine, have been shown to be inhibitors which are competitive with tyrosine.[5, 12] These compounds also lower endogenous catecholamine levels of guinea pig[11] and rat tissues,[13] and decrease norepinephrine production in patients with pheochromocytoma and hypertension.[14] A series of catechols has also been reported to inhibit tyrosine hydroxylase *in vitro* by competing with the pteridine cofactor.[5–7] *In vivo*, most of these catechols failed to give significant inhibition of heart tyrosine hydroxylase,[6] but high doses of 3,4-dihydroxyphenyl-propylacetamide (H-22/54) did inhibit *in vivo*.[6, 15]

Pyrroloisoxazoles represent a novel class of tyrosine hydroxylase inhibitors, since they are not tyrosine analogues nor do they possess a catechol moiety. The inhibition by ethyl 3-amino-4H-pyrrolo[3,4c]isoxazole-5(6H) carboxylate was found to be dependent on Fe^{2+} concentration and could be reversed by adding Fe^{2+} during the course of the incubation. These observations suggest that pyrroloisoxazoles inhibit by chelating divalent iron. It is possible, however, that the compounds inhibit by another

mechanism and that the presence of excess Fe^{2+} merely inactivates the inhibitor. The observation that the inhibitor was noncompetitive with both tyrosine and pteridine cofactor, however, suggests the mechanism of inhibition to be different from that with α-methyl-*p*-tyrosine and H-22/54.

Divalent iron has been reported to activate the enzyme,[1, 4] and the chelating agents α-α-bipyridyl and *o*-phenanthroline were found to be inhibitors.[1,4] The exact role of iron in the hydroxylation of tyrosine has not been elucidated, but metal ions have been proposed as catalysts in the activation of oxygen in this type of reaction.[16]

Since pyrroloisoxazoles appeared to complex Fe^{2+}, a study was made of the metal-complexing properties of these compounds. Mixture of CL-65263 and ferrous ions in 0·1 N HCl formed a deep red color with an absorption maximum at 510 mμ. The color was not observed with any other metals tested. Absorption spectra of CL-65263 and Fe^{2+} mixtures showed a shift toward the 510 mμ maximum as the pH was lowered, suggesting a greater tendency to form the complex at lower pH. This observation correlated well with the greater enzyme inhibition seen at lower pH (Table 5). Although these observations are parallel, other explanations for the pH effect on the enzyme inhibition must not be discounted.

In addition to ferrous ions, the inhibition of tyrosine hydroxylase by CL-65263 was prevented by copper ions. Although CL-65263 did not form a visible colored complex with either cuprous or cupric ions, the inhibitor may chelate copper through its free amine group in a manner similar to that observed with certain amino acids and amines.

Structure–activity analysis of several pyrroloisoxazoles showed that the greatest enzyme inhibition occurred when there was a free amine group at position 3. Similarly, the greatest tendency to form a metal complex occurred with compounds having this 3-amine group; this would suggest the participation of the amine group in the complexing of the metal. Modification of the group at N–5 also had an effect on the degree of enzyme inhibition. A carbonyl group at this position appeared to be necessary, with the greatest inhibition observed when the carbonyl was in an ester linkage. The alkyl esters, ethyl and isobutyl, were more active than the benzyl ester. These differences in degree of enzyme inhibition by modification of the group at N–5 were also reflected in the ability of the inhibitors to complex the metal. The tendency of the benzyl ester to complex iron was only about 30 per cent less than that of the ethyl ester. The group at position N–5 may thus contribute to the binding of the inhibitor to a site on the enzyme rather than to the formation of the metal complex.

By the use of a method for measuring tyrosine hydroxylase activity in extracts of rat adrenals, CL-65263 was found to inhibit about 50 per cent of adrenal tyrosine hydroxylase activity 3 hr after administration of 100 mg/kg. About 25 per cent inhibition was observed at a dose of 50 mg/kg. The *N*-acetylated derivative, a very weak inhibitor *in vitro*, inhibited 30 per cent at 100 mg/kg. This inhibition may be due to a deacetylation of the compound *in vivo* to form CL-65263. The direct inhibition of the enzyme in treated rats by these metal complexing agents suggests that a metal, possibly iron, participates in the hydroxylation of tyrosine *in vivo*. This would further suggest that metal-complexing agents might regulate catecholamine biosynthesis at its rate-limiting step. Both CL-65263 and its *N*-acetylated derivative were shown to lower endogenous catecholamine and serotonin levels[17] and to produce hypotension[9] in rats.

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