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TYROSINE HYDROXYLASE INHIBITION *IN VITRO* AND *IN VIVO* BY CHELATING AGENTS*

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Abstract—A variety of metal chelating agents inhibited bovine adrenal tyrosine hydroxylase *in vitro*. Bipyridyl, *o*-phenanthroline, TPTZ (2,4,6-tripyridyl-*s*-triazine) and bathophenanthroline (4,7-diphenyl-1,10-phenanthroline), which have high affinities for divalent iron, were the most effective inhibitors. Inhibition by *o*-phenanthroline was noncompetitive with tyrosine or pteridine cofactor, but dependent on iron concentration. Bipyridyl, administered to rats, inhibited adrenal tyrosine hydroxylase activity and markedly lowered adrenal, heart and brain catecholamines.

THE INITIAL step in the biosynthesis of norepinephrine and epinephrine is the hydroxylation of L-tyrosine to DOPA (3,4-dihydroxyphenylalanine). This step is catalyzed by a tyrosine hydroxylase present in brain, adrenal medulla and other sympathetically innervated tissues.[1] The enzyme has been partially purified from bovine adrenal medulla[1] and shown to require a specific pteridine cofactor.[1–3] The reported inhibition of the enzyme by 2,2′-bipyridyl[1] and *o*-phenanthroline,[3] and stimulation by ferrous ions[1, 3] suggested a metal requirement for tyrosine hydroxylation and the possible regulation of catecholamine synthesis by the use of chelating agents. The present communication describes the inhibition of tyrosine hydroxylase by a variety of chelating agents *in vitro* and evidence for the inhibition of the enzyme *in vivo* by bipyridyl.

EXPERIMENTAL

Materials. Neocuproine (2,9 dimethyl-1,10-phenanthroline), bathophenanthroline (4,7-diphenyl-1,10-phenanthroline), and TPTZ (2,4,6 tripyridyl-*S*-triazine) were obtained from G. Frederick Smith Chemical Co.; Tiron (disodium catechol-3,5-disulfonate), *o*-phenanthroline, *m*-phenanthroline, and $DMPH_4$ (2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine) from Aldrich Chemical Co.; 8-hydroxyquinoline and 2,2′-bipyridyl from Fisher Scientific Co.; *p*-bromo-*m*-hydroxybenzyloxyamine (NSD-1055) from Smith & Nephew Research, Ltd.; sodium diethyl-dithiocarbamate from J. T. Baker Chemical Co.; and EDTA (disodium ethylenediaminetetracetate) from Sigma Chemical Co. Freshly prepared aqueous solutions of ferrous ammonium sulfate were used as the source of Fe^{++}. Aqueous solutions of metal chlorides were the source of the other metal ions.

* A preliminary report of this investigation was presented at the 52nd Annual Meeting of the American Society of Biological Chemists, Atlantic City, N.J. (April 1968).

Enzyme preparation. Tyrosine hydroxylase was prepared from homogenates of beef adrenal medulla.[1] The enzyme was precipitated from the 105,000 *g* supernatant of a bovine adrenal medulla homogenate by the addition of ammonium sulfate to 40 per cent saturation. The precipitate was resuspended in 1×10^{-3}M aqueous mercaptoethanol (pH 6·5) and dialyzed overnight against 200 vol. of deionized water at 5°. The dialyzed preparation contained about 30 mg protein per ml.

Assay in vitro *of tyrosine hydroxylase.* Tyrosine hydroxylase activity was assayed by measuring the formation of tritiated water from 3,5-^{3}H-tyrosine by a modification[3] of the procedure described by Nagatsu *et al.*[4] The standard incubation mixture consisted of 0·1 μmole L-tyrosine containing 2×10^4 cpm L-tyrosine-3,5-^{3}H, 200 μmoles acetate buffer (pH 6·0), 1·0 μmole $DMPH_4$ in 0·1 ml 0·1 M phosphate (pH 7·4) containing 1 M mercaptoethanol, 0·1 μmole NSD-1055, and 0·2 ml enzyme preparation. The chelating agents were added to the incubation mixture in 0·1 ml of 50 per cent aqueous ethanol, and the final volume was adjusted to 1·0 ml with water. The chelating agents were preincubated with the enzyme and $DMPH_4$ for 10 min at 37° before addition of tyrosine, then incubated for 30 min at 37° with shaking. The reaction was stopped by the addition of 0·05 ml of glacial acetic acid and the mixture was centrifuged. The supernatant solutions were placed on small Dowex-50 (H^+) columns[3] and washed with 1·0 ml water. The effluents and washings were mixed with 10 ml Bray's solution, and radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. Controls were included to correct for non-enzymatic hydroxylation and for inhibition due to the solvent.

Estimation in vivo *of tyrosine hydroxylase.* Tyrosine hydroxylase activity was estimated in rat adrenals as described in an earlier report.[5] The chelating agents were dissolved in an appropriate solvent and administered intraperitoneally or by oral intubation to male Sprague–Dawley rats (Carworth Farms, 120–140 g) which had been fasted for 18 hr.

The animals were decapitated and the adrenals removed, freed of connective tissue and weighed. A 20 per cent (w/v) homogenate of the pair of adrenals from each rat was prepared in 0·32 M sucrose and centrifuged at 10,000 *g* for 20 min at 0°. Tyrosine hydroxylase activity was measured in 0·02-ml aliquot of the supernatant. The remaining incubation mixture contained 40 μmoles phosphate buffer (pH 6·0), 40 μmoles mercaptoethanol, 0·1 μmole $DMPH_4$ and carrier-free L-tyrosine-3,5-^{3}H ($1{\cdot}25 \times 10^{-4}$ μmoles, $2{\cdot}2 \times 10^{-4}$ cpm) in a volume of 0·05 ml. The total mixture of 0·07 ml was incubated for 20 min at 37° in a metabolic shaker and the reaction was stopped by the addition of 0·4 ml of 5 per cent (w/v) trichloroacetic acid. After centrifugation, the tritiated water formed was assayed *in vitro* by the procedure described above.

Assay of tissue catecholamines. Endogenous tissue catecholamine levels were determined on adrenal, heart and brain in certain experiments. A 0·05-ml aliquot of adrenal homogenates, used for determination of tyrosine hydroxylase activity, was mixed with 0·5 ml of 2·8 N perchloric acid and 9·5 ml of 0·01 N HCl and further homogenized using a motor-driven glass homogenizer. After 30 min, the mixture was centrifuged and aliquots of the supernatant were assayed directly for catecholamine by the fluorimetric method previously reported by Anton and Sayre.[6] Whole brain and heart norepinephrine were isolated and assayed fluorimetrically as

described by Anton and Sayre.[6] Results are expressed as micrograms per gram of tissue, using L-norepinephrine as the standard.

RESULTS

Studies in vitro. Several metal-complexing agents were tested at various concentrations for their effects on tyrosine hydroxylase. The results are shown in Table 1.

TABLE 1. INHIBITION OF TYROSINE HYDROXYLASE BY CHELATING AGENTS

Inhibitor	Concn (mM)	Inhibition (%)
o-Phenanthroline	0·01	57
2,2′-Bipyridyl	0·01	39
TPTZ*	0·01	28
Bathophenanthroline†	0·01	15
CL 65263‡	0·1	50
EDTA	0·1	35
Sodium diethyldithiocarbamate	0·1	50
8-Hydroxyquinoline	0·1	49
Sodium cyanide	1·0	15
Tiron§	1·0	6
Neocuproine‖	1·0	0
m-Phenanthroline	1·0	0

* 2,4,6-Tripyridyl-*s*-triazine.
† 4,7-Diphenyl-1, 10-phenanthroline.
‡ 3-Amino-4H-pyrroloisoxazole-5(6H)-carboxylate, ethyl ester.
§ Catechol-3,5-disulfonic acid, disodium salt.
‖ 2,9-Dimethyl-1, 10-phenanthroline.

o-Phenanthroline, TPTZ, 2,2′-bipyridyl and bathophenanthroline were the most effective inhibitors. Tyrosine hydroxylation was significantly inhibited at concentrations of 0·01 mM of these reagents, with *o*-phenanthroline giving the greatest inhibition. Diethyldithiocarbamate, 8-hydroxyquinoline, EDTA and CL 65263 inhibited at 0·1 mM. The latter compound, CL 65263, previously was reported to inhibit tyrosine hydroxylase by chelation of a cofactor metal.[5] Tiron, neocuproine, cyanide and azide inhibited only slightly or not at all at 1·0 mM. In addition, no inhibition was observed with 1·0 mM *m*-phenanthroline, which does not chelate metals.

The most potent inhibitor, *o*-phenanthroline, was selected as a representative inhibitor for studies on the mechanism of inhibition *in vitro*. Double-reciprocal plots of data indicated that the inhibition by *o*-phenanthroline was noncompetitive with tyrosine within the range of tyrosine concentration from 4×10^{-6}M to 1×10^{-4}M (Fig. 1). Similarly, the inhibition was found to be noncompetitive with $DMPH_4$ at concentrations from 5×10^{-5}M to 5×10^{-3}M (Fig. 1). Similar kinetics were earlier reported for CL 65263.[5]

As shown in Table 2, the addition of divalent iron to the incubation reversed the inhibition by *o*-phenanthroline. The inhibition produced by 2×10^{-5}M *o*-phenanthroline was almost completely reversed by addition of an approximately equimolar amount of Fe^{++}. However, no reversal of inhibition was observed when the inhibitor was α-methyl-*p*-tyrosine (2×10^{-5}M) or H-22/54 (2×10^{-4}M) by addition

of as high as 0·2 μmole Fe^{++}. α-Methyl-*p*-tyrosine and H-22/54, (3,4-dihydroxyphenylpropylacetamide) have been shown to inhibit by competition with substrate and cofactor respectively.[7]

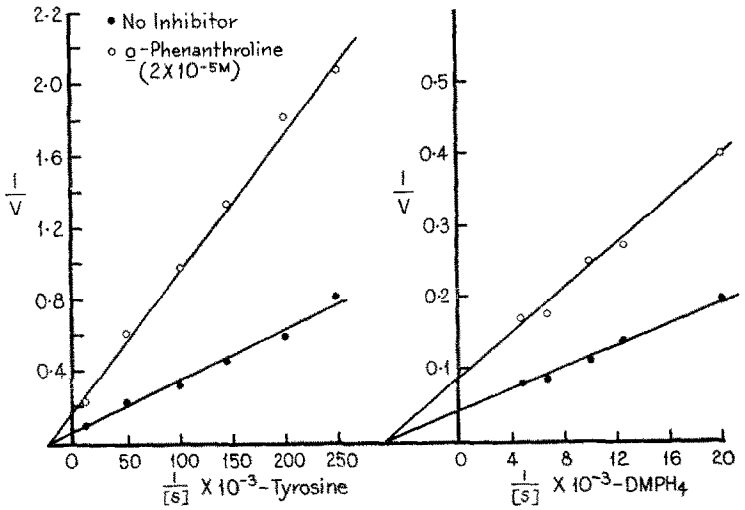

FIG. 1. Double-reciprocal plots of tyrosine or $DMPH_4$ concentration vs. rate of tyrosine hydroxylation with and without *o*-phenanthroline. The concentrations of other constituents were as described under Methods.

TABLE 2. EFFECT OF FE^{++} ON THE INHIBITION OF BOVINE ADRENAL TYROSINE HYDROXYLASE BY *o*-PHENANTHROLINE AND OTHER INHIBITORS

Fe^{++} added (μmoles)	DOPA formed (mμmoles)*			
	No inhibitor	*o*-Phenanthroline (2×10^{-5}M)	α-MpT† (2×10^{-5}M)	H-22/54‡ (2×10^{-4}M)
0	9·2	5·3	6·9	1·9
0·01	9·5	8·8	6·8	2·0
0·03	10·5	9·5	6·9	2·1
0·05	11·1	10·2	6·8	2·1
0·10	11·4	10·8	6·7	1·9
0·20	11·4	11·3	6·6	1·9

* Average of 3 determinations. *in vitro* Assay was described in Methods.

† α-Methyl-*p*-tyrosine.

‡ 3,4-Dihydroxyphenylpropylacetamide.

Studies in vivo. To determine whether chelating agents inhibit tyrosine hydroxylation *in vivo*, 2,2′-bipyridyl was administered intraperitoneally or orally to male Sprague–Dawley rats and tyrosine hydroxylase activity was determined 3 hr later in adrenal homogenates as previously described under Methods. A dose-dependent inhibition of adrenal tyrosine hydroxylase activity was observed (Table 3) in rats treated intraperitoneally or orally with 2,2′-bipyridyl. Intraperitoneal doses of 250, 100 and 50 mg/kg inhibited 76, 46 and 26 per cent respectively. Oral doses of 250, 100 and 50 mg/kg inhibited 59, 32 and 13 per cent respectively. With this method, CL 65263 inhibited 45 and 22 per cent at 100 and 50 mg/kg and α-methyl-*p*-tyrosine

TABLE 3. TYROSINE HYDROXYLASE ACTIVITY IN RAT ADRENAL EXTRACTS AFTER ADMINISTRATION OF 2,2′-BIPYRIDYL

Dose (mg/kg)	Intraperitoneal		Oral	
	Tyrosine hydroxylase activity*	Inhibition (%)	Tyrosine hydroxylase activity*	Inhibition (%)
250	2·76 ± 0·73	76	2·60 ± 0·48	59
100	6·26 ± 0·65	46	4·35 ± 0·74	32
50	8·57 ± 0·07	26	5·56 ± 0·36	13
0	11·55 ± 0·96	—	6·38 ± 0·49	—

* Expressed as per cent of ^{3}H-tyrosine hydroxylated. Initial concentration of ^{3}H-tyrosine was $1{\cdot}245 \times 10^{-4}$ μmoles/ml. Values represent average for 5 rats ± S.E. Assay *in vivo* was described in Methods.

inhibited 69 and 52 per cent at 100 and 50 mg/kg, respectively, when administered intraperitoneally.[5]

In another experiment, bipyridyl was administered intraperitoneally to rats at a dose of 100 mg/kg and the animals were sacrificed at various times after administration. Adrenal tyrosine hydroxylase activity and adrenal, heart and brain catecholamine levels were determined. Adrenal tyrosine hydroxylase activity was significantly decreased in 1 hr and continued to decrease to about 40 per cent of the control value by 6 hr (Fig. 2). Between 6 and 12 hr the activity completely recovered. Concurrent

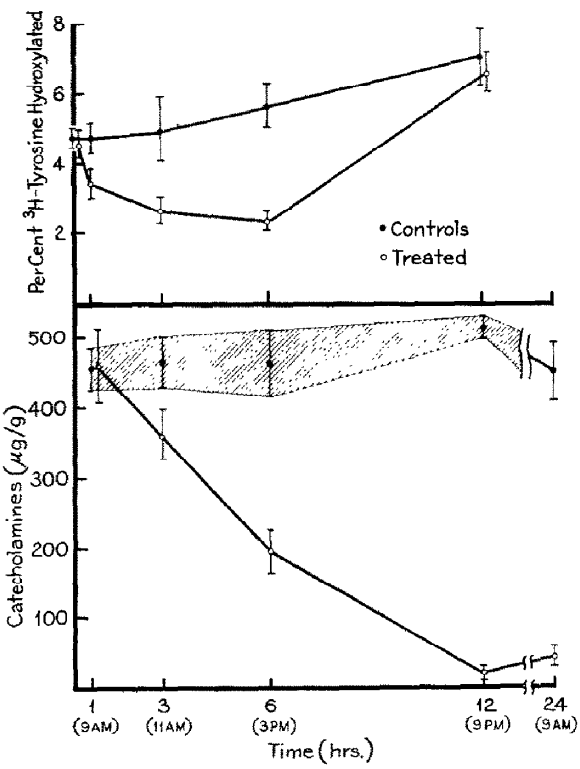

FIG. 2. Effect of 2,2′-bipyridyl (100 mg/kg) on adrenal tyrosine hydroxylase activity (top) and adrenal catecholamines (bottom). Values represent the mean for 5 rats ± standard error. Assay of tyrosine hydroxylase activity and adrenal catecholamines was described under Methods.

with the decline in adrenal enzyme activity, the adrenal catecholamine levels were decreased 22 per cent in 3 hr and continued to decrease to almost total depletion by 12 hr (Fig. 2). Only slight repletion of catecholamines was observed at 24 hr.

The effects of administration of bipyridyl on endogenous brain and heart norepinephrine levels are illustrated in Fig. 3. In brain tissues, norepinephrine levels were more

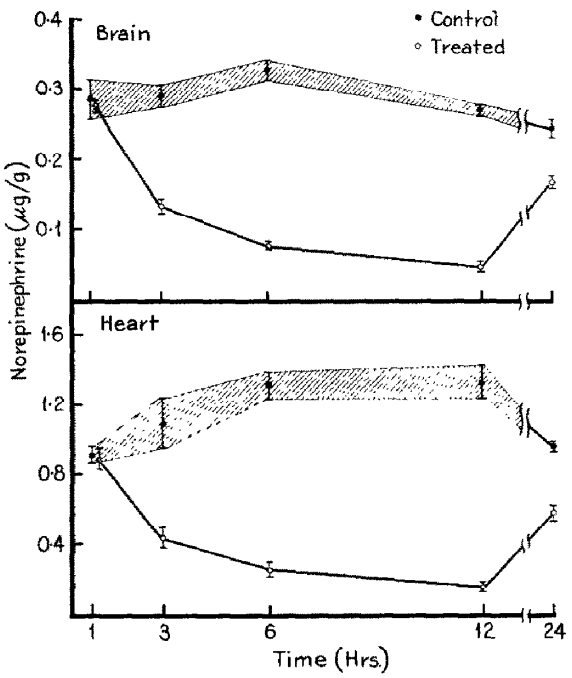

FIG. 3. Effect of 2,2′-bipyridyl (100 mg/kg) on endogenous catecholamine levels of brain (top) and heart (bottom) of rats. Values represent the mean for 5 rats ± standard error. Assay of tissue norephinephrine was described under Methods.

rapidly decreased, dropping 54 per cent within 3 hr, followed by nearly total depletion by 12 hr. Both brain and heart norepinephrine levels were significantly repleted 24 hr after treatment. All the treated animals exhibited a mild tremor and slight ptosis which completely disappeared in 24 hr.

DISCUSSION

The observed inhibition of soluble bovine adrenal tyrosine hydroxylase *in vitro* by bipyridyl[1] and *o*-phenanthroline[3] suggested a metal ion requirement for the hydroxylation. In addition, stimulation of the activity of the soluble enzyme was observed with Fe^{++}. Similarly, bipyridyl inhibition and Fe^{++} activation were observed with the tyrosine hydroxylase of the particulate fraction of adrenal homogenates.[8] These observations suggested the participation of Fe^{++} in the hydroxylation.

Previously reported inhibitors of tyrosine hydroxylase *in vitro* inhibited by substrate competition[7, 9] or by competition with pteridine cofactor.[7, 10] 3-Amino-pyrroloisoxazoles were reported to inhibit tyrosine hydroxylase *in vitro*[11] and were subsequently

found to inhibit by chelation of a metal ion.[5] These latter compounds suggested metal chelation as a third mechanism for inhibition of tyrosine hydroxylase. To further investigate this mechanism, a variety of metal chelating agents were tested for their effect on soluble bovine adrenal tyrosine hydroxylase *in vitro*. Those agents having high affinities for divalent iron, namely *o*-phenanthroline, bipyridyl, TPTZ and bathophenanthroline, were the most effective inhibitors. In addition, TPTZ and bathophenanthroline exhibit a high specificity for Fe^{++}. Tiron, EDTA and 8-hydroxyquinoline, which have high affinities for trivalent iron, were less effective inhibitors. However, this may be due to the β-mercaptoethanol in the incubation mixture, which would reduce trivalent iron to divalent iron. Diethyldithiocarbamate and cyanide, which have high affinities for copper ions, were much less effective inhibitors; and neocuproine, specific for copper, failed to inhibit. Kinetic studies indicated the inhibition by *o*-phenanthroline to be noncompetitive with tyrosine and the pteridine cofactor but dependent on Fe^{++} concentration. These observations suggest that the inhibition by *o*-phenanthroline is by metal chelation, similar to the mechanism suggested for 3-aminopyrroloisoxazoles[5] and unlike that for α-methyl-*p*-tyrosine or H-22/54. The possibility that the compounds inhibit by another mechanism and that the added iron merely inactivates the inhibitor had not been ruled out by these experiments. However, the lack of inhibition by the nonchelating *m*-phenanthroline further supports the idea of a chelation mechanism. These studies *in vitro* strongly suggest that a metal iron participates in the hydroxylation of tyrosine and furthermore the high sensitivity of the enzyme to iron-chelating agents suggests that the metal may be iron.

A dose-dependent inhibition of adrenal tyrosine hydroxylase activity was observed in rats treated with bipyridyl (Table 3) and this observation suggests the participation of a metal ion in tyrosine hydroxylation in the intact animal. Adrenal enzyme activity rapidly decreased for 6 hr after treatment with 100 mg/kg of bipyridyl (Fig. 2). Concomitantly, the adrenal catecholamine level was rapidly lowered. The observed recovery of enzyme activity between 6 and 12 hr may reflect a loss of end-product inhibition due to the decreased norepinephrine content. Endogenous norepinephrine levels have been shown to affect the rate of catecholamine synthesis *in vivo*.[12] The observed increase in adrenal enzyme activity of the untreated rats between 3 and 12 hr may be indicative of a circadian rhythmicity in the adrenal activity, the 12-hr period being 9·00 p.m.

In addition, brain and heart norepinephrine levels were markedly decreased after administration of bipyridyl (Fig. 3). These observations further suggest metal ion participation in tyrosine hydroxylation in these tissues.

This communication is the first known report of the lowering of tissue catecholamines by bipyridyl. Disulfiram, which is reduced *in vivo* to a chelating agent, has been reported to lower norepinephrine levels of heart and brain[13–15] and to inhibit dopamine-β-hydroxylase *in vivo*.[14]

An interesting observation in rats treated with bipyridyl was the development of tremor. This would suggest that the rapid lowering of brain norepinephrine levels may have been accompanied by a lowering of brain dopamine, thus causing a malfunction of the extrapyramidal system. It has been reported that iron-chelating agents significantly reduce the iron content of brain and produce choreiform movements.[16] These observations, coupled with the reported high tyrosine hydroxylase activity in

substantia nigra,[17] suggest that iron may be an important cofactor in tyrosine hydroxylation in the brain.

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