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TWO MECHANISMS FOR THE INHIBITION *IN VITRO* OF PHENYLALANINE HYDROXYLASE BY CATECHOLAMINES*

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Abstract—Norepinephrine and several related compounds inhibit the hydroxylation of phenylalanine to tyrosine in a system consisting of glucose dehydrogenase, dihydropteridine reductase and phenylalanine hydroxylase. The component sensitive to norepinephrine in this system is phenylalanine hydroxylase. The catecholamine is a competitive inhibitor of the hydroxylase with respect to the pteridine cofactor; it inhibits the hydroxylase noncompetitively with respect to phenylalanine. Norepinephrine also inhibits the hydroxylase by a time-dependent process which is prevented by reductants, aromatic amines and chelators. This progressive inhibition and the failure to accumulate a potent inhibitor from the catechol suggest that an unstable derivative, probably an *o*-quinone, formed from the catechol is strongly inhibiting the enzyme. This inhibition is partially reversed by ascorbate. Catalase does not prevent inhibition by norepinephrine. Ferrous ion and mercaptoethanol, which prevent inhibition by cumene hydroperoxide, do not prevent inhibition by norepinephrine. Oxygen is required for the inactivation of the hydroxylase by norepinephrine.

DURING a study of phenylalanine hydroxylase, it was noted that adrenal extracts inhibit the hydroxylation of phenylalanine to tyrosine. The inhibitory substances in the extracts were identified as epinephrine and norepinephrine. This paper describes two mechanisms of inhibition by catecholamines of the hydroxylase: (1) norepinephrine is a competitive inhibitor with respect to the reduced pteridine cofactor; (2) norepinephrine appears to inactivate progressively the enzyme by unstable *o*-quinones formed from the catechols.

EXPERIMENTAL

Materials. Phenylalanine hydroxylase from rat liver was purified through the second ammonium sulfate step and dihydropteridine reductase from sheep liver was purified through the alkaline ammonium sulfate step in Kaufman's procedures.[1] Glucose dehydrogenase from beef liver was prepared through the acid precipitation step of Strecker and Korkes.[2] 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine, prepared by catalytic hydrogenation[3] of 2-amino-4-hydroxy-6,7-dimethylpteridine (Aldrich), which had been recrystallized from 0·5 N HCl,[4] was stored in separate tubes at −15°.[5] The reduction was quantitative as determined by titration of the product with 2,6-dichlorophenolindophenol standardized with ascorbic acid.[6] Rat liver hydroxylation cofactor was prepared by the method of Kaufman.[1] Freshly prepared solutions of cumene hydroperoxide (Matheson, Coleman & Bell) were

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prepared by a 1:100 dilution of an aqueous solution saturated with hydroperoxide. Catalase was obtained from Calbiochem.

Methods. Incubations of a 1-ml system with the same components used by Kaufman with phenylalanine hydroxylase rate-limiting were carried out for 45 min with shaking in air at 25° unless otherwise indicated.[1] This system contains: 0·25 mM NADP; 0·25 M glucose; 0·1 M potassium phosphate, pH 6·8; 2 mM L-phenylalanine; 0·1 ml cofactor; and saturating amounts of pteridine reductase and glucose dehydrogenase. When indicated, phenylalanine hydroxylase activity was measured using dithiothreitol to regenerate tetrahydropteridine.[7] The reaction was stopped by the addition of 2 ml of 12% trichloracetic acid. Tyrosine in 2 ml of the protein-free supernatant fluid was measured colorimetrically.[8] Corrections for appropriate zero-time controls were always made. Rat liver cofactor was assayed by the method of Kaufman.[9] Protein was measured by the biuret test.[10] The kinetic data were evaluated using the computer program of Hanson *et al.*[11] The K_i was estimated using the computer program of Cleland[12] for linear competitive inhibition.

RESULTS

Effect of two concentrations of norepinephrine on the time course of the formation of tyrosine. Norepinephrine inhibits the hydroxylation of phenylalanine slightly at the onset of the reaction (Fig. 1). The proportionality of the inhibition at the beginning of the reaction to the amount of catechol added suggests that norepinephrine is directly inhibiting the reaction. In addition, the catecholamine becomes a more potent inhibitor as the reaction proceeds. The latter type of inhibition suggests either the slow inactivation of an essential component of the system by norepinephrine or

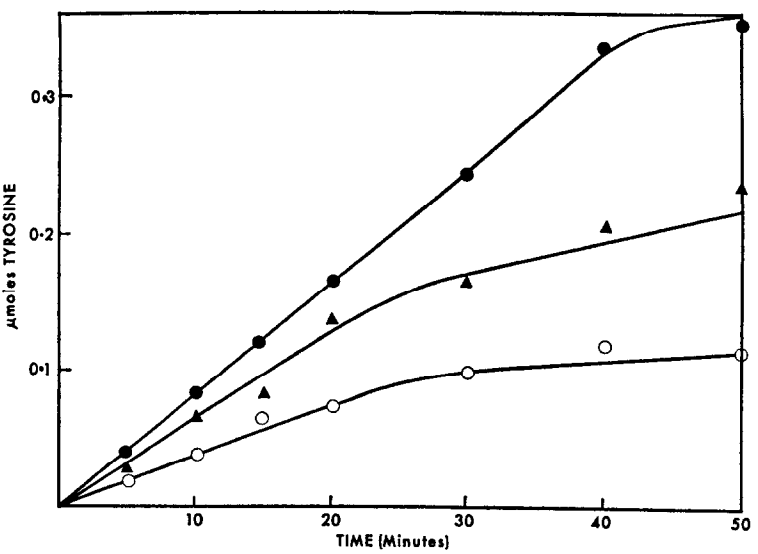

FIG. 1. Time course of formation of tyrosine in the presence and absence of norepinephrine. The experimental procedure described in the text, except the incubation period, was varied as noted. The amounts of protein added were: glucose dehydrogenase, 15 mg; pteridine reductase, 1·4 mg; phenylalanine hydroxylase, 0·24 mg. In the absence of norepinephrine, ●—●; in the presence of 0·025 mM L-norepinephrine, ▼—▼; in the presence of 0·075 mM L-norepinephrine, ○—○.

by a compound produced from the catecholamine, or the accumulation of a strong inhibitor produced from norepinephrine.

TABLE 1. COMPARISON OF THE EFFECTS OF VARIOUS ADDITIONS TO THE SYSTEM AFTER INCUBATION FOR 25 min IN THE PRESENCE AND ABSENCE OF NOREPINEPHRINE*

Incubation period (min)	Additions after 25 min	Tyrosine formed (nmoles)	
		With 100 nmoles DL-norepinephrine	Without norepinephrine
25	None	98	147
50	None	110	225
50	Glucose dehydrogenase	122	228
50	Dihydropteridine reductase	110	220
50	Phenylalanine hydroxylase	185	283
50	Ascorbate (10 μmoles)	149	297
50	Rat liver cofactor (0·10 ml)	140	310

* The experimental procedure is that described in the text, except that the incubations were carried out for the times indicated and that, after incubation for 25 min, second additions were made as noted. The following amounts of protein were added: glucose dehydrogenase, 1·5 mg; pteridine reductase, 0·89 mg; phenylalanine hydroxylase, 0·19 mg.

Effect of various additions to the inhibited system. In order to identify the component in this complex system which is inhibited by norepinephrine, several additions were made to the reaction after it had proceeded in the presence of the catechol until inhibition was nearly complete (Table 1). Additions of dihydropteridine reductase and glucose dehydrogenase were without effect. Only the addition of phenylalanine hydroxylase restored the production of tyrosine during the second 25-min incubation period to that observed during the first 25-min period. This indicates that the component of the system most sensitive to inhibition by norepinephrine is phenylalanine hydroxylase. The cofactor content of the hydroxylase was too low to have a significant role in the stimulation produced by the second addition of this enzyme. Both ascorbate and the cofactor preparation stimulate the reaction some, possibly by partially reversing the inhibitory action of norepinephrine.

Time course of the hydroxylation of phenylalanine after the addition of phenylalanine hydroxylase to the inhibited system. Further support indicating that the hydroxylase is inhibited by norepinephrine was obtained by following the time course of the reaction after addition of hydroxylase to the inhibited system (Fig. 2). Addition of the hydroxylase to the inhibited system again virtually restores the rate of tyrosine formation during the second 25-min period to that during the first 25 min. The rate of the reaction after the second addition of hydroxylase again falls progressively. These results argue against the slow accumulation of a stable inhibitor formed from norepinephrine during the incubation, since the initial rate of tyrosine formation after the addition of the enzyme to the inhibited system is essentially identical to the rate at the beginning of the reaction.

Effect of cumene hydroperoxide and ascorbate on the time course of the hydroxylation of phenylalanine. A possible explanation for the mechanism of inhibition by norepinephrine is that the catechol is stimulating the formation of an unstable peroxide which is rapidly inhibiting the hydroxylase. Accordingly, the effect of

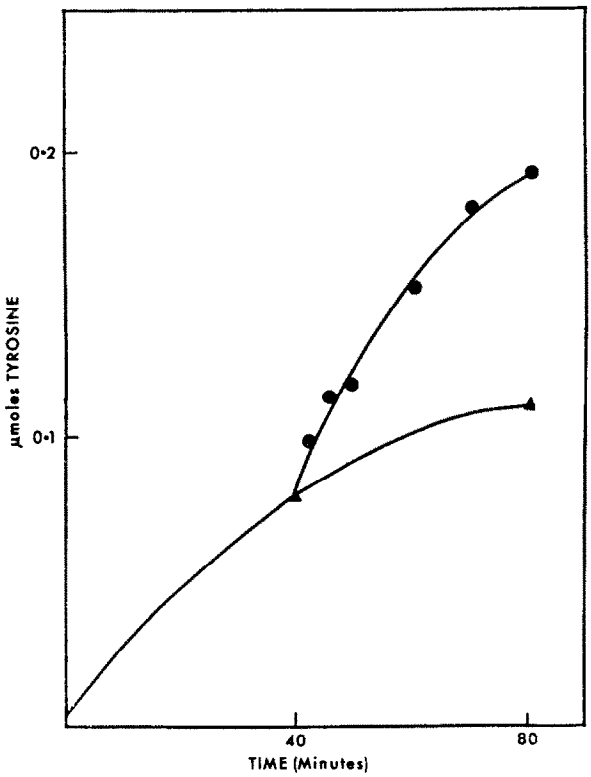

FIG. 2. Effect of the addition of phenylalanine hydroxylase to the inhibited reaction. The procedure is described in the text. The amounts of protein used are given in Fig. 1. Without the second addition of hydroxylase after 25 min of incubation, ▼—▼; with addition after 25 min of incubation of the same amount of hydroxylase added to the system at zero-time, ●—●.

cumene hydroperoxide on the reaction was tested (Fig. 3). The reaction proceeds at a linear rate for 50 min in the absence of hydroperoxide. When an amount of hydroperoxide producing about 50 per cent inhibition at 50 min is added, there is a lag period before the reaction proceeds at a rate slightly less than that observed in the absence of hydroperoxide. Thus, during the lag period there appears to be competition for a common reductant such as NADPH or reduced cofactor between the system destroying the hydroperoxide and the hydroxylase. After the apparent destruction of the hydroperoxide, tyrosine formation occurs at nearly the same rate as it does in the absence of hydroperoxide. Ascorbate prevents inhibition by the hydroperoxide, most likely by reducing the hydroperoxide.

Comparison of the specificity of reducing agents preventing inhibition by norepinephrine and cumene hydroperoxide. If the catechols are inhibiting the reaction by stimulating formation of peroxides, it would be expected that the same reducing agents which prevent inhibition by hydroperoxide would also protect against catechol, assuming a common process for the destruction of all peroxides. Hence, the abilities of various reducing agents to prevent inhibition by the hydroperoxide and norepinephrine were compared (Table 2). In the absence of reductant, both the hydroperoxide

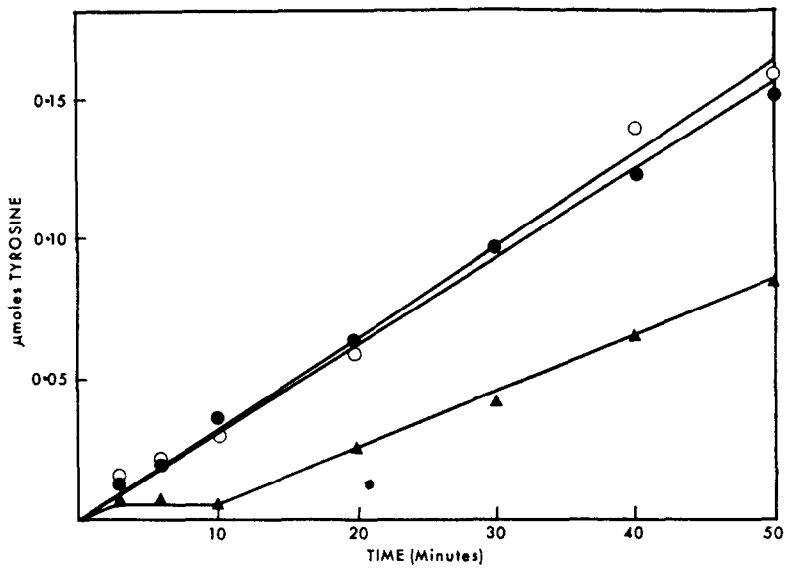

FIG. 3. Effect of cumene hydroperoxide and ascorbic acid on the time course of the hydroxylation of phenylalanine. The experimental procedure is described in the text. The amounts of protein used were: glucose dehydrogenase, 1·5 mg; pteridine reductase, 0·89 mg; phenylalanine hydroxylase, 0·19 mg. Cumene hydroperoxide (0·02 ml) added, ▲—▲; 0·02 ml cumene hydroperoxide + 10 μmoles ascorbic acid added, ○—○; no cumene or ascorbic acid added, ●—●.

and norepinephrine inhibited the reaction about 55 per cent. All the reducing agents tested, except $FeSO_4$ and mercaptoethanol, largely prevented the action of either inhibitor, though several of the reductants themselves lowered the rate of the reaction. On the other hand, the finding that mercaptoethanol and $FeSO_4$ prevented inhibition only by cumene hydroperoxide but not by norepinephrine indicates that inhibition by the catecholamines is not mediated by peroxides, which would have been destroyed by $FeSO_4$ and mercaptoethanol.

TABLE 2. COMPARISON OF THE EFFECT OF VARIOUS COMPOUNDS ON THE INHIBITION BY CUMENE HYDROPEROXIDE AND NOREPINEPHRINE*

Additions	Amount (μmoles)	Tyrosine formed (nmoles)		
		None	0·02 ml Cumene hydroperoxide	75 nmoles DL-Norepinephrine
None	—	170	79	67
L-Ascorbate	10	169	165	150
D-Isoascorbate	10	185	160	159
Cysteine	10	159	159	123
Mercaptoethylamine	10	125	114	113
$FeSO_4$	2	150	159	85
Thioglycolate	10	70	67	65
Mercaptoethanol	10	61	60	35
Thioglycolate	10	88	87	61

* The experimental procedure is described under Methods. The indicated additions were made to the incubation media. The amounts of protein added were the same as those in Table 1.

Effect of ascorbate on the specificity of inhibition. The data in Table 3 show that all catechols tested inhibit the hydroxylation of phenylalanine. Inhibition is largely lost by removal of a hydroxyl group (phenol) or by substitution of phenolic hydroxyl group by chlorine (*o*-chlorophenol). The inhibitions by all the catechols tested were prevented to some extent by ascorbate. The degree of protection by ascorbate depends on the amount of inhibition; the greater the inhibition, the more the protection by ascorbate.

TABLE 3. SPECIFICITY OF INHIBITION IN THE PRESENCE AND ABSENCE OF ASCORBATE*

Additions	Amount (*n moles*)	Tyrosine formed (nmoles)		Stimulation by ascorbate (%)
		Without ascorbate	With 10 μmoles ascorbate	
None	—	435	490	13
3-Hydroxy-4-methoxy-DL-mandelic acid	100	*345	440	27
3,4-Dihydroxyphenyl-acetic acid	100	380	468	23
Phenol	100	425	460	9
o-Chlorophenol	100	416	445	7
Pyrocatechol	100	173	351	203
DL-Norepinephrine	100	161	398	147
L-Epinephrine	100	200	370	185

* The indicated additions were made to the system described under Methods. The amounts of protein added were: glucose dehydrogenase, 1·8 mg; pteridine reductase, 1·6 mg; phenylalanine hydroxylase, 0·34 mg.

Specificity for the prevention of inhibition by norepinephrine. The data in Table 2, which show that most sulfhydryl compounds and ascorbate prevent inhibition by norepinephrine, do not provide much insight into the mechanism of inhibition. The data in Table 4 show that two other classes of compounds are also effective in the

TABLE 4. SPECIFICITY FOR PREVENTION OF INHIBITION BY NOREPINEPHRINE*

Additions	Amount (μmoles)	Tyrosine formed (nmoles)		Inhibition by norepinephrine (%)
		With 75 nmoles norepinephrine	Without norepinephrine	
None	—	173	306	44
EDTA	2·0	148	245	40
α,α′-Dipyridyl	1·0	60	84	29
Diethyldithiocarbamate	1·0	92	99	7
Aniline	10·0	189	204	7
p-Phenylenediamine	10·0	76	82	7
Hydroquinone	10·0	272	293	7
Ascorbate	10·0	262	299	12
Catalase	0·23 mg	148	300	51

*The conditions used are those described under Methods with the noted additions. Protein was added in the amounts used in Table 3.

prevention of most of the inhibition by the catechol. Of the chelators tested, diethyldithiocarbamate, which is also a reductant, is the most effective in preventing inhibition by the catechol; however, α,α′-dipyridyl is somewhat less effective and EDTA is without effect. The other class of compounds which is effective in preventing inhibition by catechols are the aromatic amines. These data, together with the failure to provide evidence for the accumulation of an inhibitor derived from catechol and the time-dependent inhibition, suggest that the catechol is oxidized to an unstable, reactive *o*-quinone, which then directly inactivates the hydroxylase. The reducing agents would reduce the *o*-quinone back to the corresponding catechol. The chelators would remove a metal ion, which would catalyze the aerobic oxidation of catechols to *o*-quinones, and the aromatic amines would remove the *o*-quinones by chemical combination.[13,14] The failure of catalase to prevent inhibition by the catechol suggests that peroxide formed during the oxidation of the catechol group does not play a significant role in the inhibition.

Requirements for the inactivation of phenylalanine hydroxylase by norepinephrine. If *o*-quinones derived from catechols are inhibiting the hydroxylase irreversibly in part, then norepinephrine should inactivate the enzyme by an aerobic process. The data in Table 5 show that inactivation of the hydroxylase by norepinephrine requires only oxygen in addition to the catechol and the hydroxylase. Omission of any of the other components listed in Table 5 did not significantly affect the stability of the hydroxylase. These results are in accord with the idea that *o*-quinones formed by aerobic oxidation from the catechols are directly inhibiting the enzyme.

Inhibition of phenylalanine hydroxylase by norepinephrine. The data in Tables 2 and 4 indicate that ascorbate prevents most, but not all, of the inhibition by norepinephrine. The ascorbate-resistant inhibition likely is due to the direct action of the

TABLE 5. REQUIREMENTS FOR THE INACTIVATION OF PHENYLALANINE HYDROXYLASE BY NOREPINEPHRINE*

Omissions during first incubation	Tyrosine formed (nmoles)	Inhibition (%)
None	22	87
Pteridine reductase	30	82
Cofactor	43	74
Norepinephrine	147	11
Glucose dehydrogenase	60	64
Phenylalanine hydroxylase	141	14
Oxygen (N_2 used)	165	0

* The complete system during the first incubation contained, in a volume of 0·6 ml: 100 μmoles potassium phosphate, pH 6·8; 0·10 ml rat liver cofactor; 0·1 μmole DL-norepinephrine; 1·5 mg glucose dehydrogenase; 2 μmole L-phenylalanine; 1·6 mg pteridine reductase; 0·48 mg phenylalanine hydroxylase. The first incubation was for 30 min at 25°. The second incubation was begun after the addition of 10 μmoles dithiothreitol, 0·4 μmole 5,6,7,8-tetrahydro-5,6-dimethylpteridine, and the components missing during the first incubation and water to 1·0 ml. The second incubation was for 20 min at 25° under air. Two ml of 12% trichloroacetic acid was added and tyrosine was measured in 2 ml of the clarified supernatant fluid.

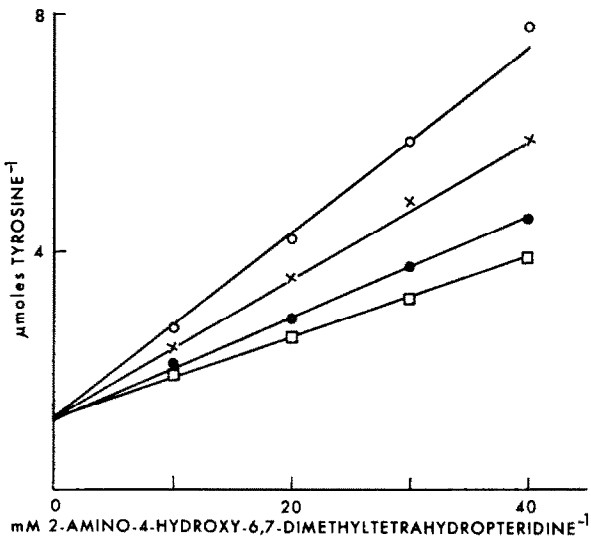

FIG. 4. Double reciprocal plots of velocity vs. 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine concentrations at various concentrations of L-norepinephrine. The conditions are those described in the text with the indicated additions of L-norepinephrine and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. The computed maximal velocities and 90 per cent confidence limits (in parentheses) are: 0·73 (0·66–0·80) μmoles tyrosine at 0 mM norepinephrine; 0·77 (0·76–0·78) μmoles tyrosine at 0·15 mM norepinephrine; 0·86 (0·81–0·92) μmoles tyrosine at 0·30 mM norepinephrine; 0·87 (0·68–1·06) μmoles tyrosine at 0·45 mM norepinephrine. No norepinephrine, □—□; 0·15 mM norepinephrine, ●—●; 0·30 mM norepinephrine, ×—×; 0·45 mM norepinephrine, ○—○.

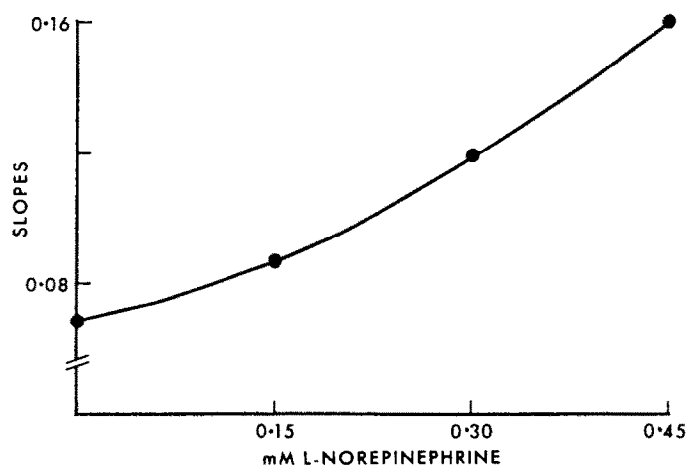

FIG. 5. Replots of slopes vs. norepinephrine concentration when 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine is the variable substrate.

catechol on the hydroxylase. Support for this idea is found in Fig. 4, which shows that the catechol is a competitive inhibitor of the hydroxylase with respect to the pteridine cofactor, since all the lines have a single vertical intercept. The inhibition constant for norepinephrine is estimated to be 0·36 ± 0·03 mM using a program for linear competitive inhibition. However, a replot of the slopes in Fig. 4 vs. the inhibitor

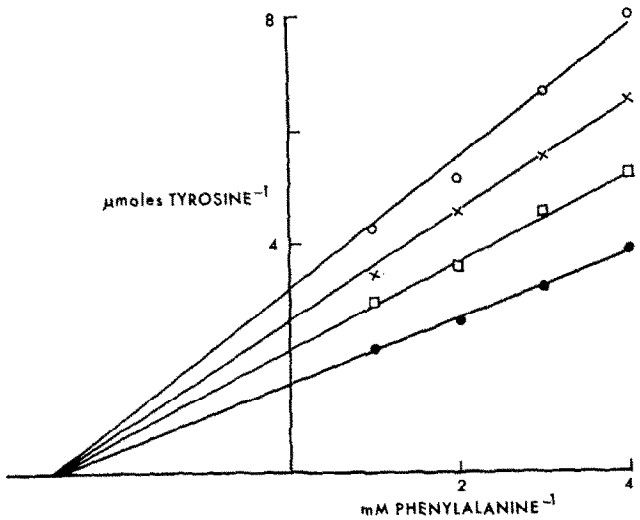

FIG. 6. Double reciprocal plots of velocity vs. phenylalanine concentrations at various concentrations of L-norepinephrine. The conditions are those described in the text except that 40 μM 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine was used and the indicated additions were made. The computed apparent K_m values with 90 per cent confidence limits (in parentheses) are: 3·10 (2·10–4·44) mM phenylalanine at 0 mM norepinephrine; 3·21 (2·02–5·09) mM phenylalanine at 0·1 mM norepinephrine; 3·85 (2·24–6·27) mM phenylalanine at 0·15 mM norepinephrine. At 0 mM norepinephrine, ●—●; at 0·05 mM norepinephrine, □—□; at 0·1 mM norepinephrine, ×—×; at 0·15 mM norepinephrine, ○—○.

concentration (Fig. 5) is not linear. The nonlinear replot shows that the mechanism of inhibition is more complex than simple competition between the reduced pteridine and the catecholamine for a common site on the enzyme. The mechanism of the inhibition was not further investigated. In this experiment the norepinephrine was dissolved with dithiothreitol (final concentration, 25 mM) in order to minimize the aerobic oxidation of the catecholamine. The data in Fig. 6 show that norepinephrine inhibits the enzyme noncompetitively with respect to phenylalanine. At the lower concentrations of norepinephrine used in Fig. 6, a replot of the slopes vs. inhibitor concentration is linear.

DISCUSSION

Norepinephrine and presumably several other catechols inhibit *in vitro* the enzymatic hydroxylation of phenylalanine to tyrosine by two mechanisms, one of which is time-dependent and sensitive to ascorbate and other reagents, and another that is insensitive at least to ascorbate. The ascorbate-resistant mechanism involves competitive inhibition by the catecholamine of the hydroxylase with respect to the reduced pteridine. Norepinephrine inhibits the enzyme noncompetitively with respect to phenylalanine. A similar type of inhibition was reported earlier by Udenfriend *et al.*[15] for tyrosine hydroxylase, which is competitively inhibited by 3,4-dihydroxyphenylpropylacetamide with respect to the pteridine cofactor and non-competitively with respect to tyrosine. At that time, Udenfriend *et al.* suggested that other pteridine-linked enzymes may be competitively inhibited by catechols with respect to the cofactor. It is possible that this type of inhibition is responsible to some extent for

inhibitions of either phenylalanine or tryptophan hydroxylations catalyzed by liver phenylalanine hydroxylase reported earlier.[16–20]

The catecholamines also inhibit phenylalanine hydroxylase by a time-dependent process requiring oxygen which is sensitive to a number of different reagents. The strongest evidence that phenylalanine hydroxylase is also the site of the slow inhibition by the catechols is that a second addition of the hydroxylase to the inhibited system fully restores the rate of tyrosine formation to its expected value (Fig. 2). Several observations suggest that this mechanism involves the conversion of norepinephrine to an *o*-quinone which then directly inhibits the hydroxylase. In the first place, this type of inhibition requires the catechol group. Secondly, reducing agents such as ascorbate and sulfhydryl compounds capable of reducing *o*-quinones to catechols, aromatic amines which combine rapidly with *o*-quinones, and chelators which may remove metal ions that catalyze the oxidation of catechols to *o*-quinones largely prevent this type of inhibition.[13,14] However, it should be pointed out that interpretations other than the one given above are possible for the data of the effects of the chelators on the enzyme and their interaction with norepinephrine. For instance, the chelators themselves may inhibit the hydroxylase by a mechanism similar to that of the postulated *o*-quinone but that the effects are not additive. Alternatively, the norepinephrine site may no longer be rate-limiting at the catechol concentrations used in the enzyme inhibited by a chelator. Furthermore, the inhibition is time-dependent with no evidence for the slow accumulation of a strong inhibitor derived from norepinephrine. The failure to accumulate such a potent inhibitor probably is due to the instability of the highly reactive *o*-quinone. Time-dependent inhibitions of other enzymes by *p*-quinones have been reported.[14] However, the time-dependency of the inhibition of the hydroxylase by norepinephrine reported here most likely is due in part to the slow oxidation of the catechols to the *o*-quinones rather than only to the slow reaction of quinones with the enzyme.

Two lines of evidence suggest that the time-dependent inhibition is not due to peroxides. One of these is that ferrous ion and mercaptoethanol do not prevent inhibition by norepinephrine while they block inhibition by cumene hydroperoxide. The other indication against a role of peroxide is that catalase does not prevent the inhibition by norepinephrine. These results strongly argue against a significant role of peroxide formed during the aerobic oxidation of the catechol in inactivating the hydroxylase.

The slow inactivation of phenylalanine hydroxylase by *o*-quinones derived from catechols may account for at least part of the inhibition *in vivo* of this enzyme by α-methyl-DOPA.[21] This enzyme seems to be more susceptible than the other two enzymes used in this system. The mechanism by which the postulated *o*-quinone inhibits the hydroxylase is obscure.

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