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Modification of Norepinephrine Synthesis in Intact Tissue by Drugs and during Short-term Adrenergic Nerve Stimulation*

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THE rate of synthesis of norepinephrine in the adrenergic neuron is markedly influenced both by adrenergic neuronal activity and by many pharmacological inputs which may only indirectly affect the enzymes which catalyze norepinephrine formation. Many substances influence norepinephine synthesis by affecting norepinephrine uptake, storage, release or catabolism.

Norepinephrine is to a large extent stored within the adrenergic neuron in vesicles, some or all of which possess dense osmiophilic cores (9–11, 49, 52). These vesicles are analogous to chromaffin granules of the adrenal medulla (4, 19), and, on nerve stimulation, the neurotransmitter presumably is released from these storage sites directly into the synaptic cleft (9). The uptake process into the storage vesicle appears to be energy dependent and requires adenosine triphosphate (ATP) and magnesium. It is selectively blocked by agents of the reserpine type (12, 25). The neuron is also able to take up and concentrate norepinephrine across the axonal membrane. This process is not blocked by reserpine but is selectively blocked by another group of agents, among which are cocaine, imipramine, desmethylimipramine and protriptyline (29, 32, 70). The axonal membrane uptake process appears to be responsible for the termination of the biological effects of released catecholamines. Block of axonal membrane uptake leads to potentiation of the effects of norepinephrine (58).

Monoamine oxidase, an enzyme localized in mitochondria (8, 48), is also present in the adrenergic neuron. This enzyme is presumably responsible for the degradation of free intraneuronal norepinephrine. If uptake into the vesicle is blocked, *e.g.*, by reserpine, the free amine is rapidly degraded by monoamine oxidase, largely within the neuron (3, 47). Catechol-O-methyl transferase, the second major enzyme responsible for the ultimate degradation of catecholamines, is also present in the adrenergic neuron, but apparently the extraneuronal enzyme, chiefly in the liver and kidney, is more important in the metabolism of catecholamines than that present within the adrenergic neuron (2).

All of the enzymes necessary for the synthesis of norepinephrine from tyrosine are present within adrenergic nervous tissue (fig. 1). After postganglionic denervation of the sympathetic nerves to the heart, this organ loses the ability to syn-

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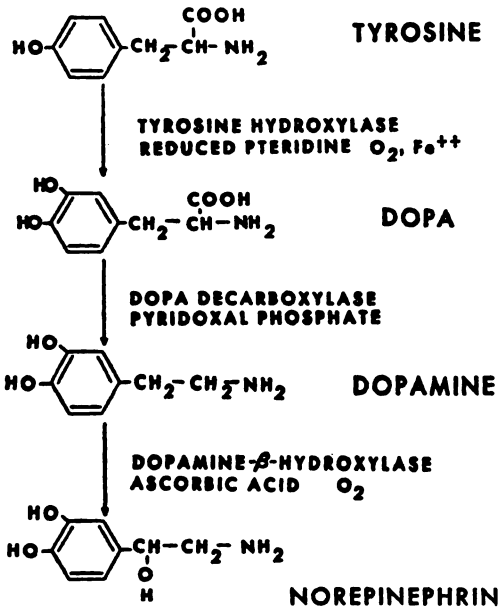

FIG. 1. The enzymatic steps in the synthesis of norepinephrine from tyrosine. (From N. Weiner: Regulation of norepinephrine biosynthesis. Annu. Rev. Pharmacol. 10: 273–290, 1970.)

thesize norepinephrine either from tyrosine or from 3,4-dihydroxyphenylethylamine (dopamine) (45). Tyrosine hydroxylase is able to catalyze the hydroxylation of both tyrosine and phenylalanine and it appears to be the rate-limiting step in the biosynthesis of the adrenergic neurotransmitter (20, 39, 51). Although there is some controversy over the intracellular localization of the enzyme, the bulk of the evidence supports the view that tyrosine hydroxylase is a soluble enzyme present in the axoplasm of the adrenergic neuron. Petrack *et al.* (43) have demonstrated that a large fraction of tyrosine hydroxylase activity in bovine adrenal medulla may be recovered in the particulate fraction of sucrose homogenates and the enzyme may be solubilized by treatment of the tissue with proteolytic enzymes. On the other hand, Musacchio and co-workers have accumulated evidence which suggests that the particulate localization of bovine adrenal medulla enzyme is an artifact resulting from the self-aggregation of tyrosine hydroxylase and the ease with which the enzyme irreversibly adsorbs to particulate structures (36, 38, 71). Results in our laboratory confirm those of Musacchio. We have demonstrated that the soluble bovine adrenal medulla enzyme will rapidly aggregate and is then easily sedimented by centrifugation. When soluble bovine adrenal medulla enzyme is mixed with membranes isolated from brain or liver, and the membranes are sedimented by centrifugation, the bulk of the enzyme is found in the sediment. This is in contrast to the soluble adrenal enzyme from a variety of other species including man, guinea pig, rat and rabbit. Tyrosine hydroxylase from the adrenals of these species is found mostly or entirely in the

cytosol and the enzyme exhibits little tendency to aggregate or to adsorb to membranes in a non-specific fashion (69).

In homogenates of sympathetic ganglia and of bovine splenic nerves, tyrosine hydroxylase appears to be largely in the soluble supernatant fraction (34, 55). Although there have been reports that a considerable fraction of brain tyrosine hydroxylase is associated with particles (22, 33), studies in our laboratory indicate that much of the particulate enzyme of brain tissue may actually be present in solution in that fraction of axoplasm which is trapped within synaptosomes. There have been reports that there may exist two types of tyrosine hydroxylase which exhibit different kinetic properties and which may be localized in different regions of the neuron (20, 23).

Tyrosine hydroxylase requires oxygen and a reduced pteridine cofactor for activity (6, 20, 39). The pteridine cofactor of bovine adrenal medulla is similar to or identical with tetrahydrobiopterin (30). Iron also appears to be required in the enzymatic hydroxylation of 3,4-dihydroxyphenyl-L-alanine (dopa) (20). Shiman *et al.* (51) suggest that iron may be involved largely in the degradation of the hydrogen peroxide which accumulates during the enzymatic reaction and which adversely affects enzyme activity. We have confirmed that iron is required for optimal activity in the tyrosine hydroxylase reaction and have demonstrated complete loss of activity in the presence of iron chelators, such as α,α-dipyridyl. Although catalase does increase the activity of tyrosine hydroxylase to some degree, it is unable to completely replace iron in the system. Our results are in agreement with Nagatsu *et al.* (39) and Petrack *et al.* (43) who claim that iron is an essential cofactor in the tyrosine hydroxylase reaction.

Kinetic studies on the mechanism of tyrosine hydroxylation suggest that during the hydroxylation of tyrosine to dopa the enzyme itself is oxidized to an inactive form. The reduced pteridine combines with the inactive, oxidized form of the enzyme, converting the hydroxylase to the active reduced form. Tetrahydropterin is simultaneously converted to the dihydro form and must be enzymatically reduced before it may function again to regenerate reduced tyrosine hydroxylase (20). Pteridine reductase, which catalyzes the conversion of dihydropteridine to the tetrahydro form, has been demonstrated in adrenal glands and appears to be distinct from dihydrofolate reductase (37). Thoa *et al.* (56) and Musacchio (37) have shown that methotrexate, a potent inhibitor of dihydrofolate reductase, does not inhibit dihydropteridine reductase.

In investigations of the tyrosine hydroxylase of beef adrenal medulla, Udenfriend and co-workers demonstrated that catecholamines and other catechols are able to inhibit the activity of tyrosine hydroxylase (39). Ikeda *et al.* (20) presented evidence that this inhibition represents competition between the catecholamine and the pteridine cofactor for the oxidized form of the enzyme. By employing a synthetic pteridine cofactor, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$), at a concentration of 10^{-3} M, Udenfriend *et al.* (59) demonstrated approximately 50 % inhibition of the activity of partially purified tyrosine hydroxylase with 10^{-3} M norepinephrine. It is quite likely that considerably smaller concentrations of catecholamines in the axoplasm of the

neuron may severely inhibit tyrosine hydroxylase activity *in situ*, since the concentration of the cofactor within the neuron is probably considerably less than 1 mM (30). Since the tyrosine hydroxylase reaction appears to be the rate-limiting step in the biosynthesis of norepinephrine, end-product feedback inhibition of tyrosine hydroxylase may be an extremely critical regulatory mechanism for the biosynthesis of catecholamines (39, 54, 62).

Decarboxylation of dopa to dopamine is catalyzed by L-aromatic amino acid decarboxylase (dopa decarboxylase) (31). Dopa decarboxylase requires pyridoxal phosphate for activity. The enzyme is located in the cytoplasm of adrenergic nervous tissue and adrenal chromaffin cells (62).

Dopamine-β-hydroxylase catalyzes the synthesis of norepinephrine from dopamine. The enzyme is a copper containing enzyme which requires oxygen and a reducing agent, ascorbic acid, as a co-substrate in the reaction (24). The enzyme is located within the storage vesicle and therefore the site of storage of norepinephrine is also the site at which norepinephrine is finally synthesized. Dopamine-β-hydroxylase is partially located within, or attached to, the membrane of the granule and a portion of the enzyme is present in the soluble contents of the storage particle (60). Uptake of dopamine into the storage vesicle is a necessary prerequisite to the hydroxylation of this substrate (26, 47).

Since tyrosine hydroxylase is the rate-limiting enzyme in norepinephrine synthesis and since its activity is affected by the end-product and by related substances (39, 54, 59), it seems obvious that this enzymatic step would be a logical site for regulation of the formation of this neurotransmitter. Any alteration in norepinephrine metabolism in the adrenergic neuron which exposes the enzyme to different quantities of end-product will lead to altered activity of the enzyme. Many pharmacological and biochemical studies have demonstrated that the hydroxylation of tyrosine is indeed extremely sensitive to a variety of physiological and pharmacological stresses which affect norepinephrine metabolism (62).

Monoamine Oxidase Inhibitors and Indirectly Acting Sympathomimetic Amines

Neff and Costa (40, 41) demonstrated that norepinephrine turnover and norepinephrine synthesis from tyrosine are reduced approximately 50% in animals treated with a monoamine oxidase inhibitor. Spector *et al.* (54) confirmed this effect of monoamine oxidase inhibitors on catecholamine synthesis from tyrosine in brain and heart *in vivo* and further demonstrated that enhanced formation of catecholamine was demonstrable when labeled dopa was employed as precursor. Both groups of investigators suggested that increased norepinephrine levels result in reduced norepinephrine synthesis as a consequence of enhanced end-product feedback inhibition of tyrosine hydroxylase. Conversely, the enhanced formation of labeled norepinephrine from labeled dopa is apparently due both to reduced dilution of the labeled pool with dopa formed from endogenous, unlabeled tyrosine and to the preservation of the catecholamines formed as a consequence of the inhibition of monoamine oxidase (54).

Weiner and Selvaratnam (67) demonstrated that tyramine reduces catechol-

amine synthesis from tyrosine *in vitro* in concentrations which do not affect the formation of catecholamines from dopa. The effect of tyramine on norepinephrine synthesis is potentiated by monoamine oxidase inhibitors. The inhibitory effect of tyramine on tyrosine hydroxylase is indirect, since tyramine, except at extremely high concentrations, has no effect on soluble tyrosine hydroxylase prepared from tissue homogenates. Other indirectly acting sympathomimetic amines which displace norepinephrine from storage sites also markedly inhibit tyrosine hydroxylation in intact tissue (27). Presumably tyramine and similar agents release norepinephrine from storage sites and tyrosine hydroxylase is inhibited in proportion to the elevation of free intraneuronal norepinephrine (fig. 2).

In contrast, when synthesis of norepinephrine from tyrosine is examined in isolated guinea pig vas deferens-hypogastric nerve preparations, the monoamine oxidase inhibitor, pargyline, fails to affect the total amount of catecholamines formed (65). This observation suggests either that monoamine oxidase inhibitors cannot influence norepinephrine synthesis from tyrosine unless the endogenous concentration of free intraneuronal norepinephrine is increased, *e.g.*, by tyramine, or that the degradation of catecholamines by monoamine oxidase is roughly equivalent to the increased synthesis of catecholamines which occurs when a monoamine oxidase inhibitor is absent. The latter explanation seems quite likely, since net catecholamine formation is considerably greater in the presence of a monoamine oxidase inhibitor than in its absence when dopa is employed as the labeled precursor (65).

Recently, we developed a tyrosine hydroxylase assay (61) which can be applied to intact tissues and which appears to be equally valid in the presence or absence of monoamine oxidase inhibitors (63). With this assay, we have been able to test directly whether monoamine oxidase inhibitors inhibit norepinephrine synthesis *in vitro* under circumstances where total tissue catecholamines are unchanged. The assay involves the use of carboxyl-labeled ^{14}C-L-tyrosine. When tissue containing tyrosine hydroxylase is incubated with carboxyl-labeled tyrosine in a 95 % O_2–5 % CO_2 atmosphere, the substrate is hydroxylated to form carboxyl-labeled dopa. Quantitative decarboxylation of carboxyl-labeled dopa occurs in intact tissue without the addition of a preparation of aromatic amino acid decarboxylase. The CO_2 which is formed is evolved at the termination of the reaction by the addition of acid to the system. The CO_2 is collected in organic base and counted by liquid scintillation spectrometry.

With this coupled decarboxylation assay for tyrosine hydroxylase, we have directly compared the formation of CO_2 from carboxyl-labeled tyrosine with the formation of catecholamines from 3,5-^{3}H-tyrosine (65) in the intact mouse vas deferens preparation (table 1). In the presence of 1.5×10^{-4} M pargyline, a concentration of this substance sufficient to inhibit monoamine oxidase virtually completely, the production of CO_2 and tritiated catecholamines is the same. This result would seem to indicate that monoamine oxidase inhibition does not affect synthesis of catecholamines from tyrosine. However, when pargyline is omitted from the incubation system, the production of CO_2 from carboxyl-labeled tyrosine is increased approximately 30 % and the production of ^{3}H-catecholamines from

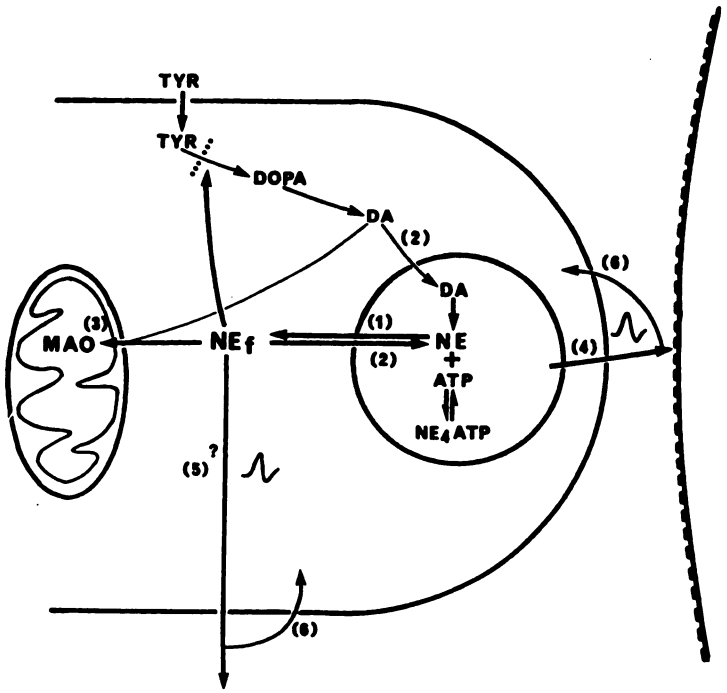

FIG. 2. Schematized depiction of the regulation of tyrosine hydroxylase by end-product feedback inhibition. Agents which would be expected to increase levels of free intraneuronal norepinephrine (NE_f) and consequently enhance inhibition of tyrosine hydroxylase activity in intact tissue include: (a) inhibitors of monoamine oxidase (MAO) (site 3); (b) reserpine alkaloids which block the uptake of catecholamines into the storage vesicles (site 2) (Reserpine also blocks norepinephrine formation from dopamine by blocking dopamine uptake into the storage vesicles.); (c) indirectly acting sympathomimetic amines, which displace norepinephrine from storage sites (site 1); (d) bretylium-like compounds which block the nerve-stimulated release of norepinephrine by inhibiting nerve terminal membrane depolarization (sites 4 and ?5). Bretylium also increases free intraneuronal norepinephrine by inhibition of MAO (site 3) and by release of the amine from storage vesicles (site 1).

Nerve stimulation may stimulate tyrosine hydroxylase activity by reducing the concentration of free intraneuronal norepinephrine, as a result of accelerated loss of the amine from the axoplasm (site 5) or by making more binding sites available in the storage vesicles (site 4). Alternatively or additionally, nerve terminal depolarization may result in an altered level of an allosteric effector which regulates tyrosine hydroxylase activity by some as yet unknown mechanism. DA = dopamine; TYR = tyrosine.

3,5-^{3}H-tyrosine is reduced approximately 25%. The latter results suggest that, in the absence of a monoamine oxidase inhibitor, approximately 40% of the ^{3}H-catecholamines formed from 3,5-^{3}H-tyrosine in the mouse vas deferens preparation is oxidatively deaminated and is not recovered in the subsequent isolation procedures which involve both alumina chromatography and Dowex 50 ion-exchange chromatography. Furthermore, the difference in the production of CO_2 in the presence and in the absence of the monoamine oxidase inhibitor suggests that tyrosine hydroxylase in the mouse vas deferens preparation is inhibited

TABLE 1

The effect of pargyline on tyrosine hydroxylase activity and catecholamine synthesis from tyrosine in intact mouse vasa deferentia

	Intact Mouse Vas Deferens (pmoles × hr^{-1} × $organ^{-1}$)	
	$^{14}CO_2$ assay (61, 63)	^{3}H-Catecholamine assay (56, 65)
+ Pargyline	47.0 ± 1.7	47.5 ± 2.4
− Pargyline	61.7 ± 3.3	35.3 ± 0.9
+ Pargyline + $DMPH_4$	70.0 ± 2.84	68.0 ± 9.6

Pargyline concentration = 1.5×10^{-4} M.
$DMPH_4$ concentration = 1×10^{-3} M.
Results are the means ± S.E. of 4 separate determinations.

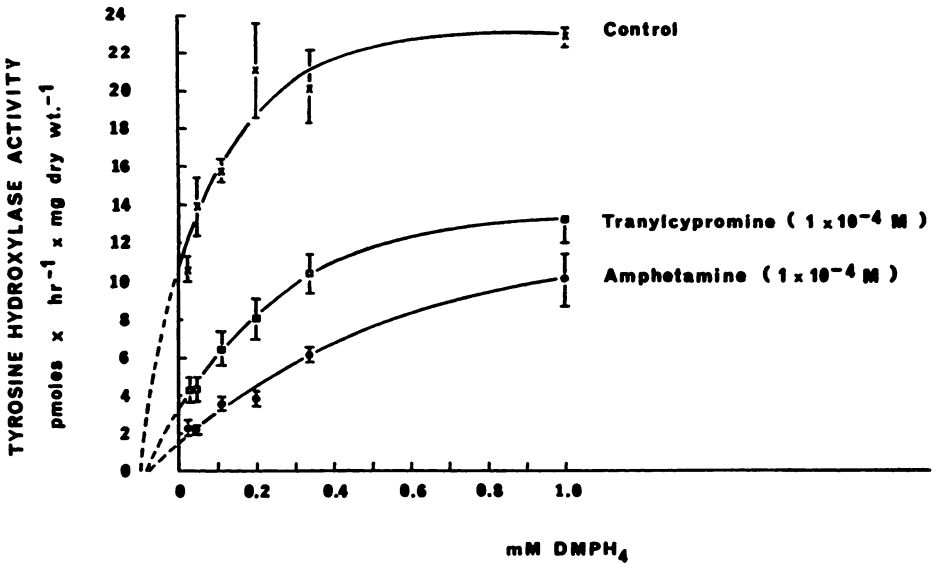

FIG. 3. The effect of 10^{-4} M amphetamine and 10^{-4} M tranylcypromine on tyrosine hydroxylase activity in intact mouse vasa deferentia at various concentrations of $DMPH_4$. Tissues were incubated in a closed tube in Krebs-Ringer bicarbonate buffer in an atmosphere of 95% O_2-5% CO_2. The buffer contained 25 mM 2-mercaptoethanol. After 15 min, the system was acidified with trichloroacetic acid and the CO_2 evolved was collected in a plastic well containing an organic base. The radioactivity was determined by liquid scintillation spectrometry. Results are the means of 4 to 6 values ± S.E. (61, 63).

approximately 30% when monoamine oxidase is inactivated, presumably as a result of enhanced end-product feedback inhibition. This presumption is supported by studies in which both pargyline and reduced pteridine cofactor are present in the system. Under these circumstances, the production of ^{3}H-catecholamines and labeled CO_2 is similar and exceeds the production of CO_2 in the absence of a monoamine oxidase inhibitor by approximately 15%. Thus, it would appear either that less than optimal amounts of pteridine cofactor are normally

present within the adrenergic neuron or that a modest degree of end-product feedback inhibition of tyrosine hydroxylase exists in this preparation even in the absence of inhibition of monoamine oxidase (table 1).

More striking reductions in the production of CO_2 from carboxyl-labeled tyrosine in intact mouse vas deferens preparations are obtained when the tissues are incubated in the presence of either 10^{-4} M tranylcypromine or 10^{-4} M amphetamine (fig. 3). The effect occurs only in intact tissue; these substances have no direct effect either on soluble tyrosine hydroxylase or on dopa decarboxylase prepared from homogenates of vasa deferentia or on partially purified tyrosine hydroxylase preparations. Since we observed that the synthetic pteridine cofactor, $DMPH_4$, stimulates the activity of tyrosine hydroxylase in intact preparations in proportion to the concentration of the pterin added to the medium (fig. 3), we attempted to determine whether the effects of tranylcypromine and amphetamine on tyrosine hydroxylase were the result of increased end-product feedback inhibition, a process which should be competitively antagonized by reduced pteridine cofactor. The effects of tranylcypromine and amphetamine on the intact mouse vas deferens preparation were examined in the presence of varying amounts of reduced pteridine cofactor. We were able to demonstrate that the inhibitory effect of these two substances on tyrosine hydroxylase could be overcome at least partially by reduced pteridine cofactor (fig. 3). A Lineweaver-Burk plot of the results suggests that reduced pteridine competitively antagonizes the effects of amphetamine and tranylcypromine at high, but not at low, concentrations of $DMPH_4$ (fig. 4). The non-linearity of the

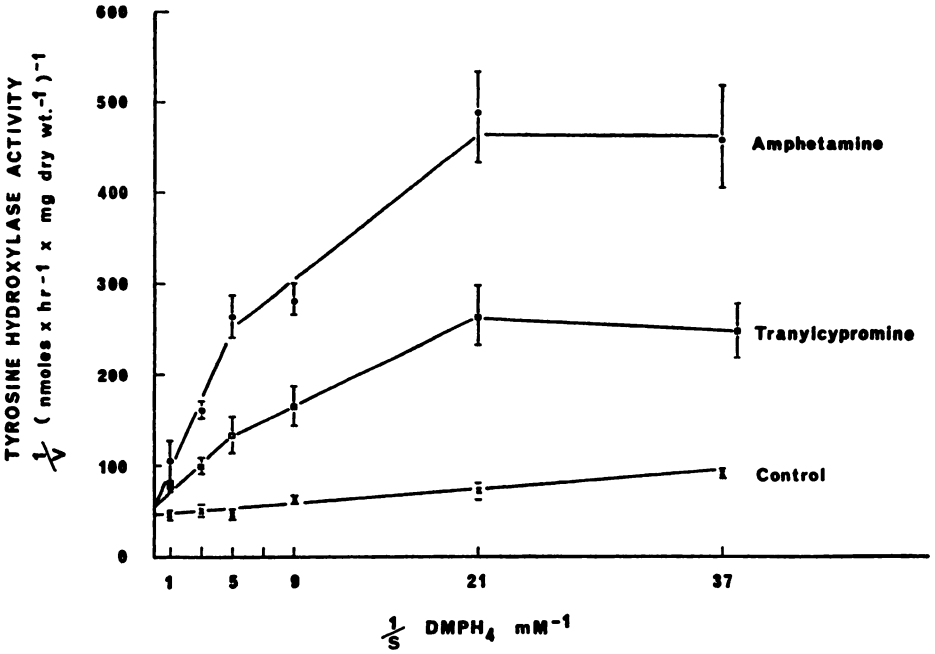

FIG. 4. Lineweaver-Burk plot of the results depicted in figure 3.

plot at low concentrations of $DMPH_4$ could perhaps be the result of the presence of significant amounts of endogenous cofactor, which are not taken into account in this analysis. If one assumes that the concentration of endogenous cofactor is approximately 0.1 mM, as is suggested by extrapolation of the hyperbolic curves relating substrate concentration to tyrosine hydroxylase activity (fig. 3), recalculation of the reciprocal of the substrate concentrations and replotting of the Lineweaver-Burk relationship provides results which suggest that the effects of tranylcypromine and amphetamine on tyrosine hydroxylase activity are competitive over the entire range of reduced pteridine cofactor concentration (fig. 5). The appropriateness of using the corrected cofactor concentration (added $DMPH_4$ + 0.1 mM) in these calculations is also defensible from a comparison of the calculated apparent K_m for $DMPH_4$ in intact vasa deferentia and in homogenates prepared from this tissue. In vas deferens homogenates, the apparent K_m for $DMPH_4$ is 2.5×10^{-4} M, a value identical to that obtained with intact preparations if the endogenous tyrosine hydroxylase cofactor concentration is assumed to be equivalent to 0.1 mM $DMPH_4$. It would thus appear that inhibitors of monoamine oxidase and agents which release norepinephrine from intragranular storage sites inhibit tyrosine hydroxylase activity in intact tissues by enhancing end-product feedback inhibition of the enzyme, an inhibitory effect which is competitive with pteridine cofactor (fig. 2).

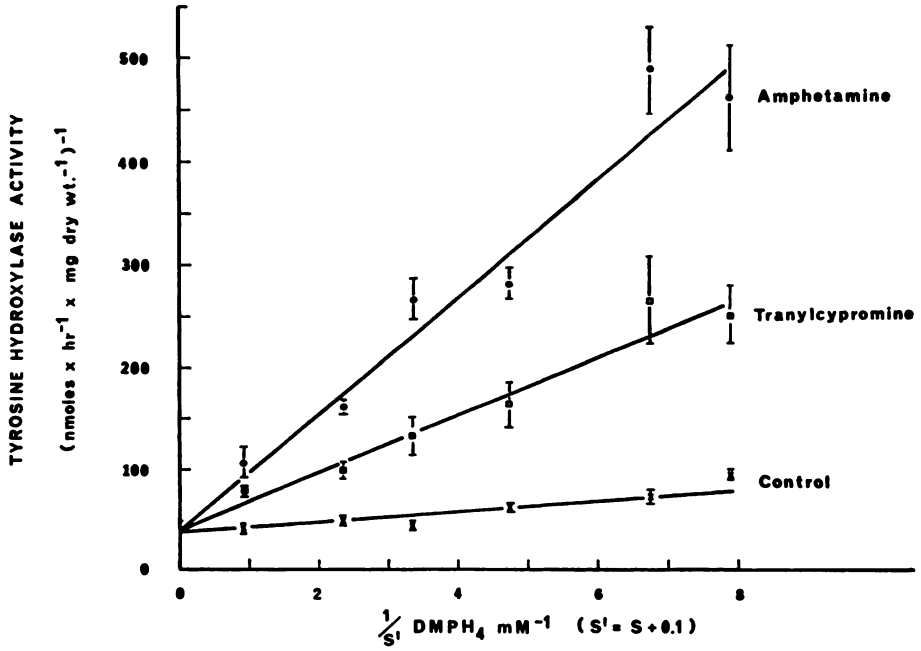

FIG. 5. Lineweaver-Burk plot of the results presented in figure 3, with a correction made for the assumed endogenous concentration of pteridine cofactor in $DMPH_4$ equivalents (0.1 mM). $DMPH_4$ concentration in the tissue is assumed to be the concentration added to the medium plus 0.1 mM.

Reserpine

Reserpine is an alkaloid which is able to block selectively the uptake of catecholamines into storage vesicles (12, 25). As a consequence of this inhibitory effect, reserpine inhibits the formation of norepinephrine from dopamine by preventing the access of the substrate, dopamine, which is formed in the axoplasm, to its enzyme dopamine-β-hydroxylase, which is present within the storage vesicle (25, 47) (fig. 2). During the acute phase of the norepinephrine depletion induced by reserpine, one would expect increased levels of free intraneuronal catecholamines which, in turn, should result in enhanced inhibition of tyrosine hydroxylase in intact tissue. After the tissues are severely depleted of norepinephrine, the end-product feedback inhibition of tyrosine hydroxylase should abate. After prolonged depletion of norepinephrine by reserpine, tyrosine hydroxylase is apparently induced, presumably as a result of reflex increase in sympathetic nervous system activity (34, 35, 57).

We have attempted to determine whether reduced activity of tyrosine hydroxylase occurs shortly after reserpine administration, by employing the coupled decarboxylation assay for intact tissue. As predicted, after the acute administration of reserpine, there is an abrupt reduction in tyrosine hydroxylase activity in intact mouse vas deferens preparations (fig. 6) and in adrenal medulla which lasts for several hours and which is followed by a gradual return of tyrosine hydroxylase activity toward normal by 24 hr. We have not been able to demonstrate a significant alteration in tyrosine hydroxylase levels in homogenates of mouse vas deferens preparations at any time up to 72 hr after daily administration of reserpine. However, in similar studies, increased levels of tyrosine hydroxylase are apparent in homogenates of adrenal glands after 3 days of reserpine administration, an effect observed earlier by Thoenen *et al.* (57) and Mueller *et al.* (34, 35). Thus, the effects of reserpine on catecholamine synthesis are extremely complex and are determined by the tissue, the degree of catecholamine depletion and the duration of the reserpine effect. Shortly after reserpine administration, the acute block of norepinephrine uptake into granules results in enhanced end-product feedback inhibition of tyrosine hydroxylase and reduced activity of this enzyme. In addition, throughout the period of this effect of reserpine, there is impaired conversion of dopamine to norepinephrine as a consequence of the inability of the substrate to enter the storage vesicle where the enzyme is located. As the levels of norepinephrine become severely depleted in the adrenergic neuron, end-product feedback inhibition of tyrosine hydroxylase subsides and, with more prolonged depletion, in some tissues one observes enhanced levels of tyrosine hydroxylase as a consequence of increased synthesis of tyrosine hydroxylase subsequent to the reflexly mediated enhancement of sympathetic nervous system activity.

Bretylium

Bretylium is a quaternary ammonium compound which exhibits profound antiadrenergic and antiarrhythmic properties. Boura and Green (5) demonstrated

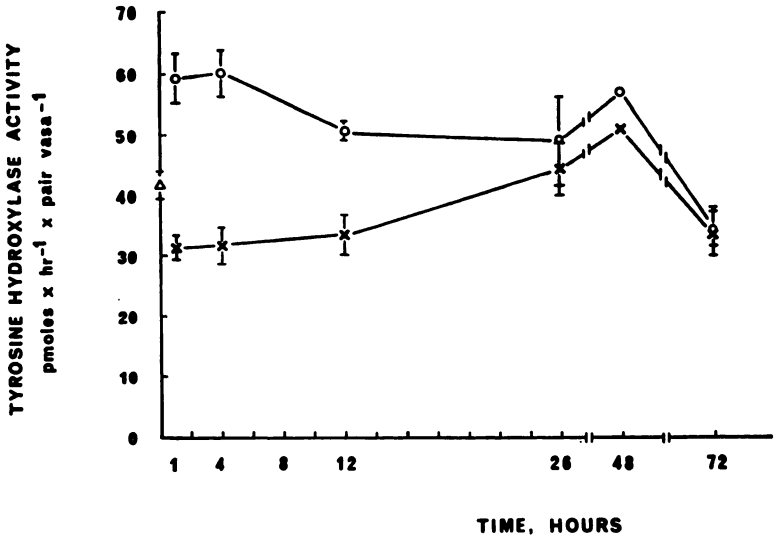

FIG. 6. Time course of the activity of tyrosine hydroxylase in intact vasa deferentia removed from mice which had received either reserpine, 2 mg/kg in 5% ascorbic acid, or an equivalent volume of the ascorbic acid vehicle, intraperitoneally at 24-hr intervals. The assay was performed as noted in the legend of figure 3 (61, 63). The tyrosine hydroxylase activity is significantly reduced in vasa deferentia of reserpine treated animals at 1, 4 and 12 hr ($P < .05$). Tyrosine hydroxylase activity in vasa deferentia of ascorbate treated controls was significantly above that of animals not treated by injection at 1 and 4 hr, presumably because of the stress associated with the injection ($P < .01$). Results are the average of 6 to 11 separate experiments ± S.E., except at the 48-hr time period, where the results presented are the means of two determinations. ×——×, reserpine treated (2 mg/kg i.p. daily); O——O, ascorbate-injected controls; △, untreated controls.

that this compound is selectively concentrated in adrenergic nerve terminals and that it inhibits electrically stimulated neuronal release of norepinephrine. Blockade of adrenergic nerve terminal depolarization should lead to reduced release of norepinephrine and markedly reduced turnover of the catecholamine in tissues. Bretylium also inhibits monoamine oxidase (14, 15, 28) and this should further compromise norepinephrine synthesis and turnover. We have found that bretylium markedly reduces norepinephrine synthesis in isolated, intact vasa deferentia, whereas the conversion of dopa to catecholamines is not profoundly affected. The inhibitory effect of bretylium on monoamine oxidase is not responsible for the marked inhibition of norepinephrine synthesis observed with this substance, since a similar inhibition of norepinephrine synthesis by bretylium is demonstrable in the presence of virtually complete block of monoamine oxidase by pargyline. The effect of bretylium on norepinephrine synthesis in intact vas deferens preparations is antagonized by excess pteridine cofactor (42) (table 2). Bretylium has no direct effect on tyrosine hydroxylase. Concentrations of this substance in excess of 1.0 mM do not inhibit partially purified tyrosine hydroxylase of guinea pig vasa deferentia.

We have attempted to evaluate the inhibition of norepinephrine synthesis by

TABLE 2

Effect of bretylium on catecholamine synthesis in intact guinea pig vas deferens preparations

Additions to Medium*	Total Catecholamine Synthesis†	
	Control	Bretylium 2.3×10^{-6} M
None	0.49 ± 0.08‡	0.05 ± 0.01
2-Mercaptoethanol 25 mM	0.20 ± 0.04	0.06 ± 0.02
$DMPH_4$ 2 mM + 2-Mercaptoethanol 25 mM	1.55 ± 0.21	0.86 ± 0.16

* Pargyline, 1.5×10^{-4} M and 7.8 mM glucose were present in the Krebs-Ringer bicarbonate medium in all experiments.

† Total catecholamines formed from 1×10^{-5} M 3,5-^{3}H-tyrosine, nmoles × g tissue^{-1} × hr^{-1}. For methods, see references 65, 66.

‡ Results are means ± S.E. of 5 separate determinations.

Ordinarily, the largest fraction of the catecholamines formed is norepinephrine. In the presence of 2-mercaptoethanol, virtually all the catecholamines are recovered in the dopaine fraction.

bretylium *in vivo* by following the rate of decline of tissue norepinephrine in rats after α-methyl-*p*-tyrosine in the presence and in the absence of bretylium according to the procedures developed by Spector (53), and Brodie *et al.* (7). Every 6 hr, animals were given intraperitoneal injections of 100 mg/kg α-methyl-*p*-tyrosine methyl ester, or 20 mg/kg bretylium tosylate, or both drugs. Norepinephrine in the heart, vas deferens and spleen was assayed fluorimetrically 24 and 48 hr after initiation of drug treatment. In those animals given α-methyl-*p*-tyrosine, a progressive fall in the concentration of norepinephrine was observed in the three tissues over the 48-hr period, although the rate of decline did not appear to follow a strictly log-linear relationship (fig. 7). Bretylium alone had no significant effect on tissue norepinephrine levels. However, bretylium reduced the α-methyl-*p*-tyrosine-induced decline in tissue norepinephrine levels during the first 24 hr and prevented any further decline in tissue norepinephrine in the second 24-hr period. These results *in vivo* are in agreement with our studies *in vitro* and indicate that the synthesis and turnover of norepinephrine are markedly reduced by bretylium and that this effect is primarily at the tyrosine hydroxylase step (44). Bretylium presumably increases the levels of free norepinephrine in the nerve ending: (a) by reducing norepinephrine release as a consequence of the blockade of nerve terminal depolarization (5); (b) by inhibition of monoamine oxidase (14, 15, 28); and (c) by release of norepinephrine from storage sites (18).

We have also demonstrated that treatment with bretylium for 48 hr results in lowered levels of tyrosine hydroxylase in homogenates prepared from animals administered this drug. Levels of tyrosine hydroxylase fall to approximately 50% of normal after 48 hr, a phenomenon which is analogous to that observed after chronic decentralization of the adrenal medulla (68).

The Effect of Acute Adrenergic Nerve Stimulation on Norepinephrine Synthesis

Increased adrenergic nervous activity after stress or exercise is associated with increased turnover of norepinephrine and increased excretion of norepinephrine

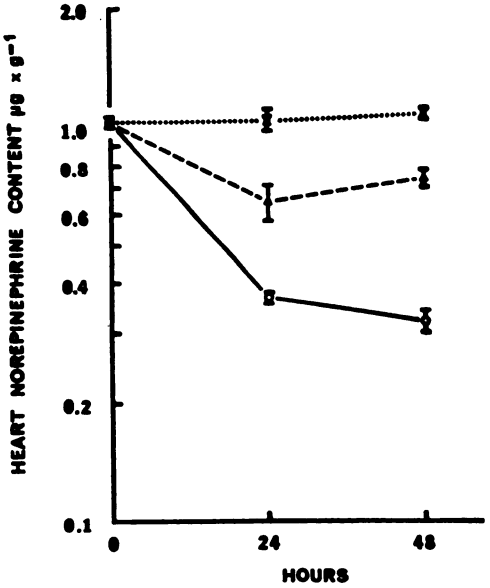

FIG. 7. The effect of bretylium and α-methyl-*p*-tyrosine on rat heart norepinephrine levels. Rats were given intraperitoneal injections of either 100 mg/kg α-methyl-*p*-tyrosine, or 20 mg/kg bretylium tosylate, or both drugs, every 6 hr. Animals were killed at 24 and 48 hr and tissue norepinephrine was assayed fluorimetrically (65). Control values are shown at time zero. Results are the mean ± S.E. of six separate determinations. ●, control; ×·····×, bretylium; ○——○, α-methyl-*p*-tyrosine; △- - -△, α-methyl-*p*-tyrosine + bretylium.

and norepinephrine metabolites in the urine (13, 62). The levels of norepinephrine in tissues of experimental animals subjected to stress remain relatively constant, thus implying that increased synthesis must occur to maintain or restore levels of neurotransmitter in the tissue. Increased adrenergic nervous activity is associated with enhanced synthesis of norepinephrine from tyrosine both *in vivo* (16, 17, 42, 50) and *in vitro* (1, 46, 65). During electrical stimulation of the hypogastric nerve-vas deferens preparation of the guinea pig, increased synthesis of ^{3}H-catecholamines from 3,5-^{3}H-tyrosine is demonstrable. This effect is not observed if dopa is employed as precursor, and suggests that the increased synthesis of norepinephrine from tyrosine results from enhanced tyrosine hydroxylase activity (65). It seems reasonable to assume that, since nerve stimulation is associated with enhanced release of norepinephrine, the increased tyrosine hydroxylase activity might be the result of reduced end-product feedback inhibition consequent to the enhanced secretion of the neurotransmitter. Norepinephrine synthesis from tyrosine in both stimulated and sham-stimulated hypogastric nerve-vas deferens preparations of guinea pigs is markedly inhibited by addition of 6×10^{-6} M norepinephrine to the medium. This inhibitory effect may be prevented by prior addition of cocaine. The enhanced catecholamine synthesis associated with nerve stimulation is at least partially reversed by exogenous norepinephrine, whereas it is not inhibited by puromycin (1, 56, 65) (table 3).

TABLE 3

Effect of nerve stimulation on norepinephrine synthesis vas deferens hypogastric nerve

	During Stimulation	Poststimulation
Tyrosine → norepinephrine	↑ ~ 50% (1, 65)*	↑ ~ 200% (56, 64)
Dopa → norepinephrine	Unchanged (65)	Unchanged or ↑ slightly (66)
TH Levels†	Unchanged‡	Unchanged (56, 64)
Inhibited by:		
Norepinephrine 6×10^{-6} M during stimulation	At least partially (1, 65)	No (66)
Puromycin	No (56)	Yes (56, 66)
Tyrosine during stimulation	No (65)	No (64)
Cycloheximide		No (56, 64)
Actinomycin D		No (56)
Bretylium		Yes (44)

* Numbers refer to references at end of this report.

† Tyrosine hydroxylase levels assayed in a fortified system in homogenates prepared from stimulated vasa deferentia or sham-stimulated preparations (56, 61).

‡ Unpublished observations.

In addition to the increased synthesis which occurs during nerve stimulation of the isolated vas deferens preparation, there is enhanced incorporation of tyrosine into norepinephrine in the immediate poststimulation period. This effect is not prevented by addition of norepinephrine to the bath during nerve stimulation. Although it is blocked by puromycin in concentrations which inhibit protein synthesis, it is not inhibited by either cycloheximide or actinomycin D. Furthermore, there is no change in the levels of tyrosine hydroxylase in the tissue during the poststimulation period, although the activity of tyrosine hydroxylase in the intact tissue is increased more than 2-fold at this time. The mechanism for this poststimulation increase in norepinephrine synthesis has not been clarified (table 3) (56, 64, 66).

The increase in norepinephrine synthesis during nerve stimulation appears to be at least partially the result of reduced end-product feedback inhibition since it is to some extent inhibited by addition of norepinephrine to the medium. The exogenous norepinephrine presumably reduces the concentration gradient across the axonal membrane so that, with nerve stimulation, there is less net loss of norepinephrine and less reduction in the level of free intraneuronal norepinephrine in the axoplasm. Under these circumstances one would expect a less marked increase in tyrosine hydroxylase activity with nerve stimulation since the end-product feedback inhibition would be less suppressed (fig. 2).

We have attempted to evaluate this possibility further by examining the effect of norepinephrine on the increase in norepinephrine synthesis during nerve stimulation more completely and by determining the effect of $DMPH_4$ on norepinephrine synthesis associated with nerve stimulation. Norepinephrine in increasing concentrations causes a progressive reduction of norepinephrine syn-

thesis from tyrosine in the intact vas deferens. The effect is more prominent in the stimulated preparation. Probit analysis indicates that the ID50 for the intact, quiescent preparation is 4.5×10^{-5} M norepinephrine, whereas the ID50 for the stimulated preparation is only 1×10^{-5} M norepinephrine. With concentrations exceeding 10^{-6} M norepinephrine, there is no longer a significant increase in norepinephrine synthesis associated with nerve stimulation (fig. 8).

If the reduction in free intraneuronal norepinephrine is responsible for the increase in norepinephrine synthesis during nerve stimulation and if the mechanism for this effect involves reduced end-product feedback inhibition by competition with the pteridine cofactor, the effect should be abolished by addition of a sufficient quantity of pteridine cofactor to the medium. Analogous to the results observed with tranylcypromine and amphetamine, the added cofactor should eliminate the end-product feedback inhibition in both stimulated and control preparations and, if this mechanism is indeed competitive, the differences between stimulated and control preparations should disappear at sufficiently high concentrations of added pteridine.

Contrary to the results predicted on the basis of the end-product feedback inhibition hypothesis, no abolition of the differences between control and stimulated preparations is demonstrable, although increased norepinephrine synthesis in both stimulated and control preparations was observed in proportion to the

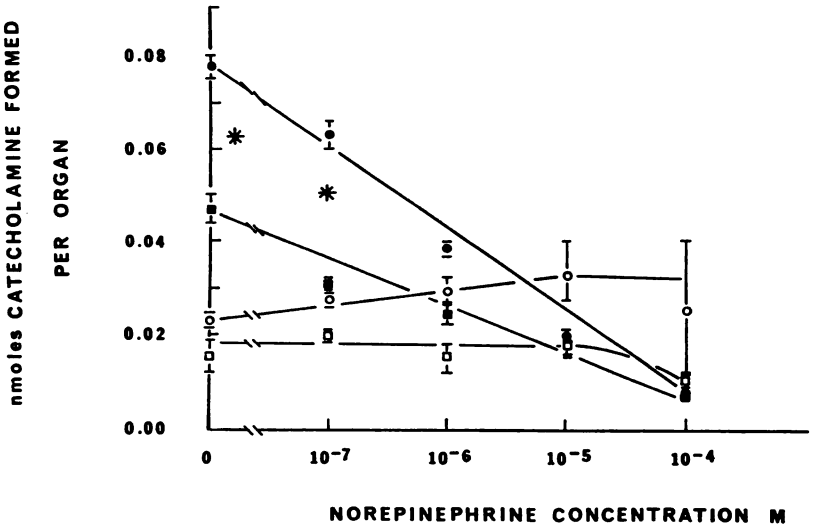

FIG. 8. The effect of nerve stimulation on catecholamine synthesis from 3,5-^{3}H-L-tyrosine in the isolated hypogastric nerve-vas deferens preparation of the guinea pig in the presence of different concentrations of norepinephrine. Experiments were performed in the presence of 1×10^{-5} M tyrosine and 1.5×10^{-4} M pargyline, as previously described (65). A significant effect of nerve stimulation on catecholamine synthesis is demonstrable only in the preparations exposed either to no exogenous norepinephrine or to 10^{-7} M norepinephrine *, ($P < .05$). ●, norepinephrine, stimulated; ■, norepinephrine, control; ○, dopamine, stimulated; □, dopamine, control.

amount of $DMPH_4$ added to the medium (table 4). Lineweaver-Burk analysis of the effect of $DMPH_4$ on norepinephrine synthesis indicates that, whereas norepinephrine competitively antagonizes the stimulatory effect of the reduced pteridine cofactor on norepinephrine synthesis (apparent K_m is increased and V_{max} is unchanged), $DMPH_4$ does not reduce or eliminate the difference in norepinephrine synthesis between stimulated and sham-stimulated preparations (apparent K_m is unchanged but the V_{max} of the stimulated preparation is approximately twice that of the control) (table 5). It would thus appear that the increased synthesis of norepinephrine from tyrosine associated with acute nerve stimulation is not

TABLE 4

Effects of $DMPH_4$ on nerve-stimulated increase in norepinephrine synthesis guinea pig vas deferens

$DMPH_4$ Added	n*	pmoles Catecholamine Formed per Organ		Increase due to Stimulation
		Control	Stimulated	
M				%
0	8	98 ± 13†	130 ± 17†	32
10^{-4}	4	106 ± 6†	228 ± 31†	116
10^{-3}	4	141 ± 5	276 ± 18	95
10^{-2}	2	324‡	873‡	169
In Presence of 10^{-6} M Norepinephrine				
0	4	42 ± 6	66 ± 10	57
10^{-4}	4	75 ± 7	136 ± 2	83
10^{-3}	6	123 ± 25	241 ± 4	96
10^{-2}	4	174 ± 15§	270 ± 24§	55

* Number of experiments.

† Values significantly higher than corresponding values in presence of 10^{-6} M norepinephrine.

‡ Tissues contracted spontaneously and responded poorly to nerve stimulation.

§ Tissues contracted spontaneously, but responded well to nerve stimulation.

Tissues were incubated for 1 hr in Krebs-Ringer bicarbonate buffer in an atmosphere of 95% O_2-5% CO_2. The medium contained 1×10^{-5} M 3,5-^{3}H-L-tyrosine, 1.5×10^{-4} M pargyline, 2 mM ascorbic acid, ± $DMPH_4$ and ± norepinephrine, as noted. Catecholamine synthesis in tissues and baths was determined as previously described (56, 65).

TABLE 5

Kinetic analysis of the effects of norepinephrine and $DMPH_4$ on the increased synthesis of norepinephrine during nerve stimulation

	Apparent K_m $DMPH_4$	Apparent V_{max} $DMPH_4$
Stimulation *vs.* control	Similar	↑ 2-Fold
Control + NE* *vs.* control	↑ > 2-Fold	Similar
Stimulation + NE *vs.* stimulation	↑ > 2-Fold	Similar
Stimulation + NE *vs.* control + NE	Similar	↑ 2-Fold

* NE, norepinephrine.

simply the consequence of reduced end-product feedback inhibition which is competitive with the pteridine cofactor. It may result from reduced inhibition of the enzyme as a consequence of reduced levels of free intraneuronal norepinephrine, but the effect may be more significantly at an allosteric site on the enzyme or at some site where the interaction is not competitively antagonized by pterin cofactor. Alternatively or additionally, some as yet unknown positive (or negative) allosteric effector may be either produced (or released or degraded) during nerve stimulation to increase tyrosine hydroxylase activity.

Summary

Monoamine oxidase inhibitors, indirectly acting sympathomimetic amines, reserpine and bretylium, which possess no direct inhibitory effect on tyrosine hydroxylase, inhibit tyrosine hydroxylase in intact, adrenergically innervated tissue, presumably by increasing the concentration of free intraneuronal norepinephrine. The increased free norepinephrine in the axoplasm inhibits tyrosine hydroxylase by competitive antagonism with the pteridine cofactor. The effects of monoamine oxidase inhibitors, indirectly acting sympathomimetic amines, and bretylium on tyrosine hydroxylase can be antagonized by addition of excess pteridine cofactor to the medium. Stimulation of the hypogastric nerve of the isolated vas deferens preparation of the guinea pig is associated with increased tyrosine hydroxylase activity both during stimulation and in the immediate poststimulation period. The poststimulation increase in norepinephrine synthesis is not antagonized by norepinephrine. The increase in tyrosine hydroxylase activity demonstrable during nerve stimulation is partially antagonized by norepinephrine, but is not reversed by the addition of excess reduced pteridine cofactor to the medium.

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