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# DRUG-INDUCED INHIBITION OF NORADRENALINE SYNTHESIS IN VITRO IN BOVINE SPLENIC NERVE TISSUE

BY

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According to recent observations during studies of noradrenaline biosynthesis in different fractions of a bovine splenic nerve homogenate (Stjärne & Lishajko, unpublished) only the β-hydroxylation of dopamine requires the presence of the granule fraction, while the ring hydroxylation of tyrosine and the subsequent decarboxylation of dihydroxyphenylalanine (dopa) proceed in the absence of granules. The present experiments were intended to study the effect on noradrenaline biosynthesis *in vitro* of various drugs known to affect noradrenaline uptake, storage, release and metabolism, *in vivo* and *in vitro*.

## METHODS

Fresh bovine splenic nerve tissue was obtained from the slaughter house and homogenized at 0°–5° C by means of an Ultra-Turrax apparatus. The whole nerve homogenate was suspended in ice-cold isotonic potassium phosphate *p*H 7.5 and centrifuged at 9,000×*g* for 10 min. The low speed supernatant, containing the specific noradrenaline-storing nerve granules (preparation 1, granules) was centrifuged at 50,000×*g* for 30 min. The high speed supernatant (preparation 2, supernatant) was used for some incubations. The high speed sediment was resuspended in potassium phosphate (preparation 3, resuspended granules).

*Incubations:* The different fractions, in volumes of 8 ml., were incubated in stoppered plastic centrifuge tubes at 20° C for 60 min in the presence of tritium-labelled tyrosine (New England Nuclear Corp., 1-tyrosine-3.5-$H^3$, specific activity 4 C/mM), 1 μC/ml. or of tritium-labelled dopamine (New England Nuclear Corp., 3.4-dihydroxyphenylethyl-1-$H^3$-amine-HBr, specific activity 50 mC/mM), 1 μC/ml. In some of the tyrosine experiments tetrahydrofolate, 5 mM, was added as fortifier of the tyrosine hydroxylase. In most cases adenosine triphosphate, 3 mM, and magnesium sulphate, 3 mM, were also added.

*Drug additions:* The experimental tubes were as a rule preincubated with the different drugs for 30–60 min at 2° C. In some reserpine experiments the drug was added simultaneously with the enzyme substrates. Drugs were used in the following molar concentrations:

Reserpine (reserpine phosphate lyophilized, Ciba) $10^{-5}$M, prenylamine (Segontin gluconate, Hoechst) $3\times10^{-5}$M, phenoxybenzamine (Smith, Kline & French) $10^{-4}$M, hydergin (Sandoz) 10 μg/ml. (about $2\times10^{-5}$M), propranolol (Inderal, ICI) $3\times10^{-4}$M, pargyline (Eutonyl, Abbotts Lab.) $10^{-4}$M.

*Analysis:* After the incubation period the different fractions were extracted with ice-cold perchloric acid and fractionated by ion exchange chromatography, using Amberlite CG120, Type 2 (200–400 mesh), as described by Stjärne & Lishajko (unpublished). The positions of the added carrier noradrenaline, dopamine and dopa were determined by reading the spontaneous fluorescence at 335 mμ (excitation wavelength 285 mμ) in an Aminco-Bowman spectrophotofluorimeter. The radioactivity of the different fractions was measured by counting 0.5 ml. aliquots of the effluent in a 7:3 toluene-

absolute ethanol solution containing 4 g. of 2.5-diphenyloxazole and 100 mg 1.4-*bis*-2(4-methyl-5-phenyl-oxazolyl)-benzene/l. toluene. The counting time was 10 min.

*Recovery:* The overall recovery of material passed through the entire procedure ranged between 70 and 90%.

## RESULTS

1. *Reserpine, prenylamine:* After preincubation of the low speed supernatant (preparation 1) with reserpine or prenylamine, the formation of dopamine from tritiated tyrosine was not affected, while the formation of noradrenaline was completely abolished.

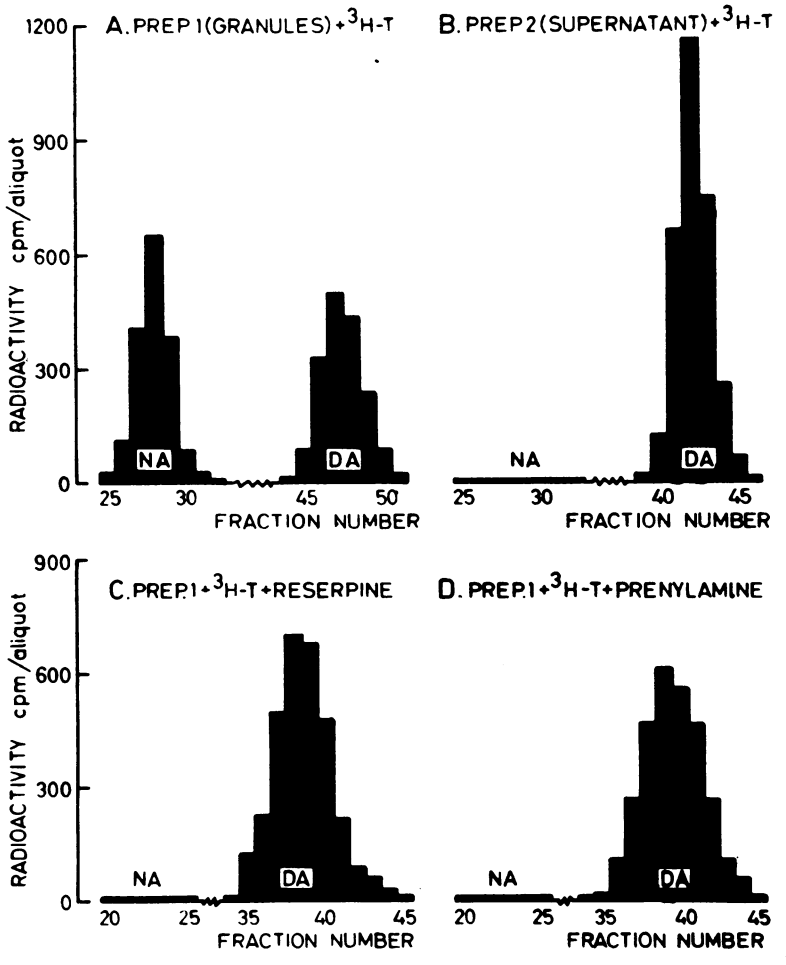


Fig. 1. Amine formation from tyrosine (T) in the granule-containing low speed supernatant and in the particle-free high speed supernatant. Inhibitory effect of drugs on noradrenaline (NA) synthesis. Ion exchange chromatograms of extracts of whole tubes after the following incubations: A. Prep. 1 (low speed supernatant) incubated with labelled tyrosine. No drug addition. B. Prep. 2 (high speed supernatant) incubated with labelled tyrosine. No drug addition. C. Same as A. Preincubated with reserpine $10^{-5}$M. D. Same as A. Preincubated with prenylamine $3\times10^{-5}$M.

The amount of newly formed dopamine found in the presence of these drugs was equal to the sum of the dopamine and noradrenaline formed in the absence of the drugs, and to the dopamine formed from tyrosine on incubation with the particle-free high speed supernatant (preparation 2, Fig. 1).

However, on incubation of the low speed supernatant (preparation 1), or of the resuspended granules (preparation 3) with tritiated dopamine, in the presence of 25-272 nmol/ml. of non-labelled dopamine, the inhibitory effect of reserpine on noradrenaline formation was found to be incomplete, even after preincubation with the drug (Fig. 2, 3). The yield of noradrenaline formed in the resuspended granules in the absence of drugs was about one-third of that in the low speed supernatant.

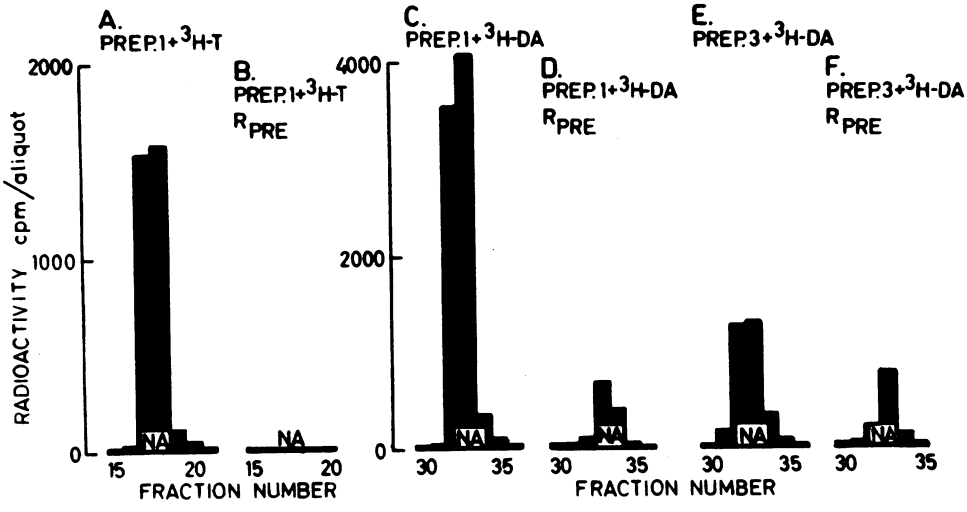


Fig. 2. Inhibitory effect of reserpine on noradrenaline (NA) formation from tyrosine (T) or dopamine (DA) in the low speed supernatant and in resuspended granules. Ion exchange chromatograms of extracts of whole tubes after the following incubations: A. Prep. 1 (low speed supernatant) incubated with labelled tyrosine. B. Same, after preincubation with reserpine $10^{-5}$M. C. Prep 1 (low speed supernatant) incubated with labelled dopamine. D. Same, after preincubation with reserpine $10^{-5}$M. E. Prep. 3 (resuspended granules) incubated with labelled dopamine. F. Same, after preincubation with reserpine $10^{-5}$M.

2. *Adrenaline antagonists:* After preincubation of the low speed supernatant (preparation 1) with phenoxybenzamine or propranolol, the dopamine formation from typosine was not affected, while the noradrenaline formation was strongly, but not completely, blocked. Hydergin (a mixture of hydrogenated ergot alkaloids) did not inhibit dopamine or noradrenaline formation from tyrosine (Fig. 4).

3. *Inhibition of monoamine oxidase:* Preincubation of the low speed supernatant (preparation 1) with pargyline at a concentration which completely inhibited the monoamine oxidase (Roth & Stjärne, unpublished) did not appreciably affect dopamine or noradrenaline formation from tyrosine. Blocking the monoamine oxidase did not change the inhibitory effect of reserpine or phenoxybenzamine on noradrenaline formation from dopamine.

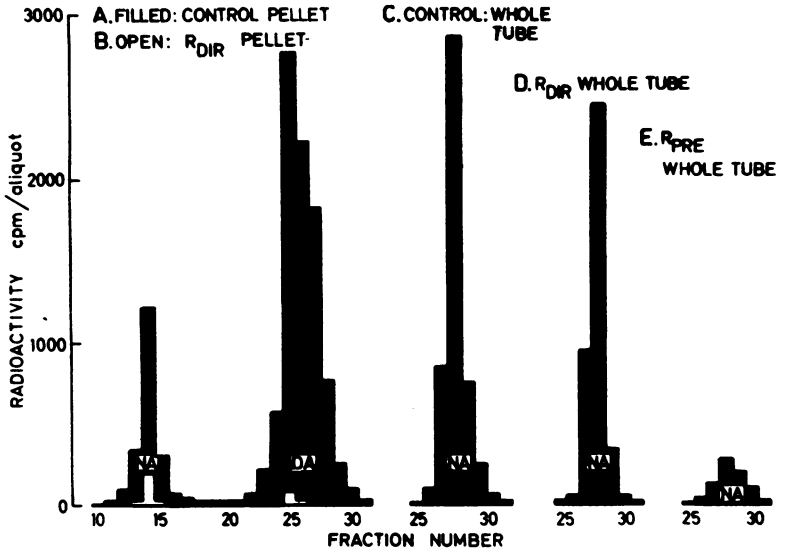


Fig. 3. Inhibitory effects of reserpine on noradrenaline (NA) formation and on noradrenaline and dopamine (DA) uptake and binding in the granules. Comparison of the effects of adding the drug simultaneously with the substrate, with those of preincubating the medium with the drug before substrate addition. A. Prep. 3 (resuspended granules) incubated with tritium labelled dopamine 25 nmol/ml. No drug addition. Ion exchange chromatogram of the sediment pellet. B. Same, on addition of reserpine $10^{-5}$M simultaneously with the labelled dopamine. Ion exchange chromatogram of the sediment pellet. C. Similar to A, but noradrenaline peak from ion exchange chromatogram of the whole tube. No drug addition. D. Similar to B, but noradrenaline peak from ion exchange chromatogram of the whole tube. Reserpine $10^{-5}$M added simultaneously with the dopamine. E. Prep. 3 (resuspended granules) preincubated with reserpine $10^{-5}$M before addition of the labelled dopamine. Noradrenaline peak from ion exchange chromatogram of the whole tube.

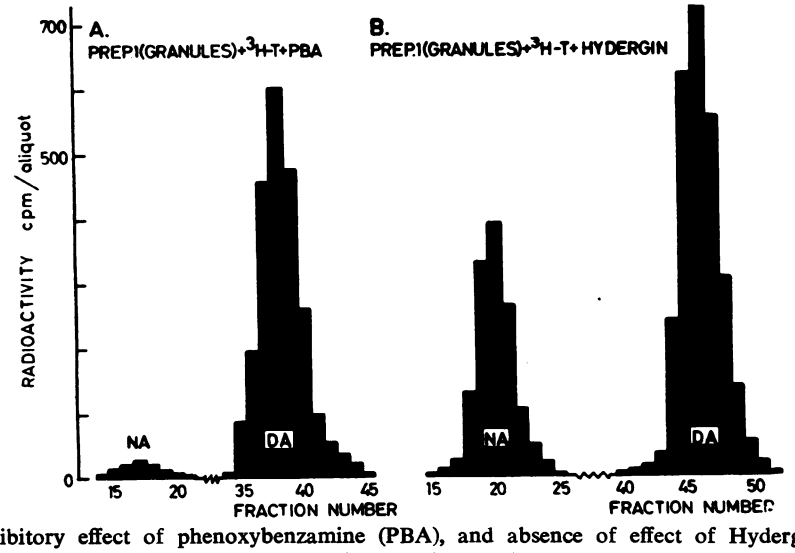


Fig. 4. Inhibitory effect of phenoxybenzamine (PBA), and absence of effect of Hydergin on noradrenaline (NA) formation from tyrosine (T) in the low speed supernatant. Ion exchange chromatograms of whole tubes. A. Prep. 1 (low speed supernatant) incubated with labelled tyrosine after preincubation with phenoxybenzamine $10^{-4}$M. B. Same preparation, preincubated with Hydergin 10 $\mu$g/ml. (about $2 \times 10^{-5}$M).

## DISCUSSION

The present experiments demonstrate *in vitro* effects on noradrenaline synthesis of a series of drugs known to interfere with noradrenaline uptake, storage, release and metabolism, *in vivo* and *in vitro*. All the drugs, except pargyline, have been shown to inhibit strongly spontaneous noradrenaline release from isolated bovine splenic nerve granules *in vitro*, at the concentrations used in the experiments (Euler & Lishajko, 1965 ; Euler & Lishajko, 1966). Most of the drugs have also been shown to block the uptake of exogenous noradrenaline into the nerve granules, at the same concentrations (Stjärne, 1964 ; Euler & Lishajko, 1966).

After preincubation of the low speed supernatant with reserpine or prenylamine the formation of dopamine from tyrosine was not affected, while that of noradrenaline was completely inhibited. Thus these drugs appear to interfere with noradrenaline synthesis exclusively at the $\beta$-hydroxylation step. In view of their inhibitory effect on amine uptake into isolated nerve granules (Euler & Lishajko, 1963 ; Stjärne, 1964), it appears probable that they inhibit noradrenaline formation from dopamine by interfering with dopamine transport rather than by an inhibitory effect on the enzyme reaction itself (cf. Kirshner, Rorie & Kamin, 1963 ; Weiner & Rutledge, 1966).

While inhibition of the last step in noradrenaline synthesis was found to be complete on incubation of the low speed supernatant with labelled tyrosine after preincubation with reserpine, the inhibitory effect was less complete when dopamine had been added to the incubation mixture. This apparent discrepancy might partly be due to the competitive nature of the reserpine-induced inhibitory effect on amine uptake in the granules (Stjärne, 1964 ; cf. Carlsson, Hillarp & Waldeck, 1963). The reserpine concentration used in the present experiments probably was high enough to prevent the dopamine molecules gradually formed from the added tyrosine from penetrating into the $\beta$-hydroxylation sites, while on sudden addition of large amounts of labelled and unlabelled dopamine the inhibitory effect of reserpine might be more easily overcome.

The incompleteness of the inhibitory effect of reserpine on the noradrenaline formation from dopamine was even more striking in experiments with resuspended granules. In this preparation the yield of noradrenaline formed from dopamine was markedly lower than in the low speed supernatant, even in the absence of inhibitory drugs. Incomplete resuspension might possibly account for this difference, preventing dopamine from penetrating efficiently to the $\beta$-hydroxylation sites. It also appears likely that the high speed supernatant, which in fact is a potassium phosphate dilution of the natural environment of the granules, might contain one or more cofactors necessary for optimum noradrenaline synthesis. Loss of dopamine-$\beta$-hydroxylase by leakage into the medium apparently does not occur, since the high speed supernatant in all the experiments has turned out to be devoid of such enzyme activity.

When reserpine was added to the resuspended granules simultaneously with the dopamine, the formation of noradrenaline was hardly affected at all. Even preincubation of the resuspended granules with reserpine resulted in an incomplete inhibition of noradrenaline formation. However, in these experiments the retention in the granules of the newly formed noradrenaline, and the uptake and retention of exogenous noradrenaline, must have been inhibited by about 95%. This suggests the existence of more than one amine uptake mechanism in the present preparation, located in one or more types of

granule, one of which might be specifically concerned with uptake of dopamine to the β-hydroxylation sites and another with recapture of endogenous noradrenaline spontaneously given off and with uptake of exogenous noradrenaline. The present experiments show that under special circumstances these two mechanisms do not appear equally sensitive to the inhibitory effect of reserpine on amine uptake. The reason for this might be topographic, involving differences in diffusion distance, making transport to the β-hydroxylation sites particularly difficult after incomplete resuspension of the granules. However, this does not seem to be the only explanation, since differences in the degree of the reserpine-induced inhibition of noradrenaline formation and of noradrenaline and dopamine uptake and retention were observed even in experiments with the low speed supernatant. Thus it appears that there exists a "true" difference in sensitivity to the (transport ?) inhibiting effect of reserpine, in the two amine uptake mechanisms involved in the present experiments.

The difference in the degree of inhibition of noradrenaline synthesis, caused by reserpine, in the granules incubated in their original medium (low speed supernatant) and in potassium phosphate (resuspended granules) with the same amounts of dopamine suggests the possibility that the original medium might contain some unknown factor which could facilitate the penetration of the drug to the strategic points in the preparation and thus "potentiate" the inhibitory effect of reserpine on noradrenaline formation.

Striking differences in inhibitory effect on noradrenaline synthesis were found between the different adrenergic blocking agents tested, at the concentrations used. Thus phenoxybenzamine and propranolol, at concentrations known to inhibit strongly both spontaneous noradrenaline release and exogenous noradrenaline uptake by isolated nerve granules ($3 \times 10^{-4}$M), almost completely inhibited noradrenaline formation, while dopamine formation was not affected at all. However, Hydergin, at a concentration of 10 μg/ml. (corresponding to about $2 \times 10^{-5}$M), which has been shown to counteract even more strongly spontaneous noradrenaline release, while less efficiently inhibiting noradrenaline uptake by the isolated nerve granules (Euler & Lishajko, 1966), did not appreciably affect the β-hydroxylation of dopamine. The possibility that this difference between the adrenaline antagonists might be due to the difference in molar concentrations used remains to be investigated. However, the present findings provide further evidence of differences in sensitivity to drugs between different amine transport mechanisms in the granules. Thus Hydergin, a representative of the classical adrenaline antagonists of the ergot alkaloid series, at a concentration which has been shown to inhibit strongly the transport mechanism responsible for spontaneous noradrenaline release on incubation *in vitro*, only moderately blocked noradrenaline uptake and did not affect the mechanism transporting dopamine to the β-hydroxylation site.

The absence of potentiation of noradrenaline formation from tyrosine by inhibition of monoamine oxidase suggests that an adequate proportion of the dopamine formed, presumably extragranularly, was protected against monoamine oxidase by being rapidly taken up and retained in the granules. This observation is in agreement with that of Levitt, Spector, Sjoerdsma & Udenfriend (1965), that inhibition of monoamine oxidase in the isolated perfused rat heart did not affect noradrenaline formation from tyrosine or dopamine.

Several of the drugs tested in the present experiments are known as noradrenaline "depleters." The results obtained *in vitro* support the view that inhibition of nor-

adrenaline synthesis at the dopamine-$\beta$-hydroxylation step may be part of the explanation of the prolonged noradrenaline depletion caused by several of these drugs *in vivo* (cf. Kirschner, Rorie & Kamin, 1963). In view of the well-known counteraction of the reserpine-induced noradrenaline depletion *in vivo*, caused by monoamine oxidase inhibition (Carlsson, Rosengren, Bertler, and Nilsson, 1957; Pletscher, Besendorf, Bächtold and Gey, 1959) it appears to be of additional interest that the reserpine-induced inhibition of noradrenaline formation from tyrosine in the low speed supernatant was not affected by blocking the monoamine oxidase.

SUMMARY

1. Isolated bovine splenic nerve granules incubated with radioactively labelled tyrosine or dopamine were used to study the inhibitory effect on noradrenaline biosynthesis of various drugs known to inhibit amine uptake into the granules.

2. Reserpine and prenylamine were found to inhibit selectively noradrenaline formation without affecting dopamine synthesis.

3. The adrenergic blocking agents phenoxybenzamine and propranolol showed the same general pattern, but inhibited noradrenaline formation less efficiently, while Hydergin, at the concentration tested, was not inhibitory.

4. Inhibition of monoamine oxidase did not appreciably affect noradrenaline formation from tyrosine.

5. Preincubation was required to produce a maximum reserpine-induced inhibition of noradrenaline formation. Noradrenaline formation was only incompletely blocked, even at reserpine concentrations which strongly inhibited the uptake and retention in the granules of dopamine and of newly formed noradrenaline.

6. This is regarded as evidence of more than one amine transport mechanism in the granule preparation, with different sensitivies to drug inhibition.

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