REGULATION IN THE CENTRAL NOREPINEPHRINE NEUROTRANSMISSION INDUCED *IN VIVO* BY *ALPHA* ADRENOCEPTOR ACTIVE DRUGS[1]

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ABSTRACT

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The level of the two major norepinephrine metabolites, 3-methoxy-4-hydroxyphenylglycol (MOPEG) and 3,4-dihydroxyphenylglycol (DOPEG), was estimated in the central nervous system of rats to study receptor-mediated regulation of release *in vivo* as reflected in biochemical changes. The norepinephrine receptor stimulating drug clonidine (0.02–0.5 mg/kg) decreased the level of endogenous total MOPEG. The accumulation of ^{3}H-MOPEG and ^{3}H-DOPEG was decreased by clonidine (0.5 mg/kg) regardless of whether ^{3}H-tyrosine or ^{3}H-dopamine was used as precursor of ^{3}H-norepinephrine. In contrast to clonidine, the two *alpha* adrenoceptor blocking drugs, phenoxybenzamine (20 mg/kg) and aceperone (20 mg/kg), induced an increase in endogenous total MOPEG and also an increase in ^{3}H-MOPEG and ^{3}H-DOPEG regardless of the precursor used. These results indicate that clonidine decreases the release of norepinephrine *in vivo* and that phenoxybenzamine and aceperone increase the release of norepinephrine. Clonidine inhibited completely the effect of phenoxybenzamine or aceperone on endogenous MOPEG. On the contrary, it was not possible to block completely the effect of small doses of clonidine by pretreatment with either phenoxybenzamine, yohimbine (2 mg/kg) or a high dose of aceperone. These results indicate that clonidine may act on a different target than the *alpha* adrenoceptor blocking drugs. *In vitro* experiments with occipital cortex synaptosomes did not indicate a direct effect of clonidine on tyrosine hydroxylation in noradrenergic nerve terminals.

In the central nervous system, *alpha* adrenoceptor antagonists have been shown to increase norepinephrine turnover (Andén *et al.*, 1967, 1972; Dairman *et al.*, 1968; Meek and

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Neff, 1973). On the contrary, the *alpha* adrenoceptor agonist clonidine has been shown to decrease central norepinephrine turnover (Andén *et al.*, 1970).

In response to nerve stimulation, phenoxybenzamine *in vitro* induces an increase in the overflow of norepinephrine in both peripheral and central norepinephrine-innervated tissues. Several explanations for the increased overflow have been suggested, including inhibition of norepinephrine binding to the receptors (Brown and Gillespie, 1957), inhibition of neuronal

uptake (Thoenen *et al.*, 1964), inhibition of extraneuronal uptake (Iversen and Langer, 1969) and increased release of norepinephrine in response to *alpha* adrenoceptor blockade (Enero *et al.*, 1972; Häggendal *et al.*, 1972; Starke and Altmann, 1973; Farnebo and Hamberger, 1974; Langer, 1974). The idea of increased norepinephrine release is further supported by the finding that also the granular enzyme dopamine β-hydroxylase is found in increasing amounts in the overflow together with norepinephrine (Johnson *et al.*, 1971; Potter *et al.*, 1971). In contrast to phenoxybenzamine, exogenous norepinephrine and clonidine induce a decrease in the overflow of stored norepinephrine in response to nerve stimulation (Starke and Altmann, 1973; Farnebo and Hamberger, 1974).

The aim of the present investigation was to analyze biochemically the nature of *alpha* adrenoceptor-induced changes in norepinephrine release in the brain *in vivo*. Preliminary studies have indicated that measurements of norepinephrine metabolites may be relevant for such studies (Carlsson and Lindqvist, 1963; Bræstrup, 1974) and we have here investigated the rat brain level of total endogenous 3-methoxy-4-hydroxyphenylglycol (MOPEG) and also ³H-MOPEG and ³H-3,4-dihydroxyphenylglycol (³H-DOPEG) after administration of ³H-dopamine or ³H-tyrosine.

The results obtained are compatible with the view that phenoxybenzamine and the other *alpha* adrenoceptor blocking drug, aceperone, increase norepinephrine release in the central nervous system (CNS) whereas the release is decreased by clonidine. Combined treatment with clonidine and phenoxybenzamine or aceperone did not indicate that clonidine acted on the same receptors as the *alpha* adrenoceptor antagonists.

Methods

Male Wistar rats of 250 to 300 g were housed individually in cages with free access to food and water. After various pretreatments, the rats were killed by a blow on the head and the whole brain, including cerebellum, was removed within 1 minute for biochemical analysis. ³H-dopamine (10 μCi) was injected intraventricularly under light ether anaesthesia according to Bræstrup *et al.* (1974). ³H-tyrosine was prepurified by passage over Al_2O_3 and a Dowex 50X4 column before reconstitution in Merlis solution, and 50 μCi in 15 μl were injected intraventricularly.

Endogenous norepinephrine, dopamine and MOPEG. Dopamine and norepinephrine were isolated by alumina adsorption (Anton and Sayre, 1962) and quantified by fluorimetry (Weil-Malherbe, 1971).

Endogenous total MOPEG was assayed by the method of Bræstrup (1973) except that $ZnSO_4$ replaced perchloric acid for precipitation of the aryl-sulfatase enzyme and that pyridine was introduced as a stabilizer. Brains were homogenized in 0.5 M acetic acid and almost neutralized with NaOH. After centrifugation, the supernatant was divided into two parts, one for total MOPEG and one for occasional estimations of homovanillic acid and 3,4-dihydroxyphenylacetic acid (Bræstrup *et al.*, 1975). Conjugated MOPEG in approximately 7 ml of supernatant was hydrolyzed with 50 μl of arylsulfatase/β-glucuronidase (Calbiochem, Los Angeles, Calif., containing 5.75 I.U. of sulfatase per ml at 30°C) at 37°C, pH 5.6, overnight. The enzyme was precipitated by 0.5 ml of 0.6 M $ZnSO_4$ followed by 100 μl of 5 M NaOH, pH 7.0 to 7.5. After 15 minutes at 0°C, the precipitated zinc hydroxide and proteins were removed by centrifugation at 18,000 × g for 5 minutes. After centrifugation, MOPEG was extracted into ethyl acetate. Vanillic mandelic acid methyl ester was added as a reference compound and pyridine (1 mg) was added as a stabilizer prior to evaporation to dryness. The dry residue was reacted with pentafluoropropionic anhydride (Fluka, AG) in ethyl acetate and the sample was reconstituted in ethyl acetate after removal of excess reagent by N_2. Gas chromatographic analysis was carried out on a 2.5% OV 17 column with methane-argon as carrier gas. Quantification was performed by the peak height ratios of the ⁶³Ni Electron Capture Detector responses and all values were corrected for recovery of 71.3%.

³H-Norepinephrine and metabolites after intraventricular injection of ³H-dopamine. This procedure was previously described in detail (Bræstrup *et al.*, 1974). In short, ³H-norepinephrine and ³H-dopamine were adsorbed onto an Amberlite CG 120 cation exchange column and eluted in two fractions with 1 M HCl. In separate experiments, determination of ³H-normetanephrine was carried out. ³H-norepinephrine and ³H-dopamine were first adsorbed onto Al_2O_3 and ³H-normetanephrine was then separated from non-catechols and residual ³H-norepinephrine and ³H-dopamine by elution in 1-ml fractions of 1 M HCl from an Amberlite CG 120 column.

Free plus conjugated ³H-MOPEG and ³H-DOPEG, which appear in the effluent from the Amberlite column, were incubated overnight with Glusulase to hydrolyze conjugates. Free ³H-MOPEG and ³H-DOPEG were then extracted into ethyl acetate and applied on cellulose-powder plates after evaporation and reconstitution in a small volume of ethyl acetate. The plates were developed in chloroform–acetic acid–water (2:2:1) and after visualizing of the carriers with

diazotized sulfanilic acid, the area containing ³H-MOPEG and ³H-DOPEG was transferred to counting vials. Tritium was measured 24 hours after addition of 10 ml of Insta-Gel to each vial.

³H-Norepinephrine and its metabolites after intraventricular ³H-tyrosine. Catecholamines and norepinephrine metabolites were estimated ½ and 2 hours after intraventricular ³H-tyrosine (50 μCi) by a method described in detail by Nielsen (1976).

Brain tissue was homogenized in acetic acid according to the method of Bræstrup et al. (1974). Norepinephrine and dopamine in the brain extract were adsorbed onto alumina according to Anton and Sayre (1962). The supernatant and 5 ml of water washing of the alumina containing ³H-tyrosine, free plus conjugated ³H-MOPEG and conjugated ³H-DOPEG were used for further analysis (see below). After washing the alumina with another 5 ml of water, ³H-norepinephrine and ³H-dopamine were eluted by shaking the alumina with 5 ml of 0.5 M acetic acid containing 0.4 mg of EDTA and 0.5 mg of ascorbic acid for 10 minutes. After separating the supernatant, this procedure was repeated with shaking for 5 minutes and the two eluates were combined. The alumina eluate was adjusted to pH 6.5 and passed through a 12 × 0.34 cm column of Amberlite CG 120 (Na⁺, pH 6.5). After rinsing with 15 ml of water, 12 ml of 0.1 M phosphate buffer, pH 6.5, and 5 ml of water, the amines were eluted with 1 M HCl. The first 4 ml were discarded and norepinephrine was eluted in the next 8 ml; four 1-ml fractions were then taken to assess separation and dopamine was eluted in 17 ml of 1 M HCl. One-milliliter portions of each fraction were used to measure radioactivity by scintillation counting.

Isolation of ³H-tyrosine, ³H-MOPEG and ³H-DOPEG. The supernatant and water washing from the alumina were titrated to pH 2 and then passed through a 12 × 0.34 cm Amberlite CG 120 column (H⁺, pH 2). After washing the column with 5 ml of water and 4 ml of 0.5 M HCl. ³H-tyrosine was eluted with 10 ml of 4 M HCl. Two milliliters of this fraction were used to estimate the radioactivity by scintillation counting.

Free plus conjugated ³H-MOPEG and conjugated ³H-DOPEG were isolated from the combined effluent, and water washing from the Amberlite CG 120 column, just used to isolate ³H-tyrosine. After adding 1.5 ml of 1 M acetic acid, the sample was adjusted to pH 5.6 to 5.7, 200 μl of Glusulase were added and the mixture was incubated at 37°C for 18 to 22 hours. After hydrolysis, the pH was adjusted to 7 and the neutral metabolites (MOPEG and DOPEG) were extracted three times with 3 volumes of ethyl acetate. The ethyl acetate was evaporated to dryness at 37°C under reduced pressure. The metabolites were redissolved two times in 1 ml of ethyl acetate containing 10 μg of MOPEG and 20 μg of DOPEG and applied in 7-cm

strips on cellulose MN 300 powder plates. The plate was saturated for 30 minutes above the solvent system before developing in chloroform-acetic acid-water (2:2:1) for 1 hour. The region corresponding to the R_f value of DOPEG (R_f = 0.09) was scraped off and placed in a counting vial. Because of a high level of radioactive interference near the R_f value of MOPEG, the thin-layer chromatographic separation of MOPEG was more complex. A 2 × 7 cm strip of cellulose powder was transferred to a glass tube from the area around the R_f value of MOPEG (0.60) after development in the solvent system chloroform-glacial acetic acid-water (2:2:1). In addition, strips 5 mm wide were always taken from the regions immediately in front of and behind the 2 × 7 cm strip and transferred to glass tubes before the plate was sprayed with diazotized sulfanilic acid. This enabled all of the area corresponding to the position of a MOPEG marker to be determined. ³H-MOPEG was extracted from the cellulose powder into 3 ml of methanol by shaking for 30 minutes. A 2-ml portion of the methanol solution containing ³H-MOPEG was applied to a thin-layer cellulose plate and rechromatographed in the solvent system n-butanol-methanol-1 M formic acid (3:1:1). After development for approximately 3 hours, the plate was dried and MOPEG was visualized with diazotized sulfanilic acid (R_f = 0.75). ³H-MOPEG was scraped off and placed in a counting vial. ³H-MOPEG and ³H-DOPEG in counting vials were measured by scintillation counting after addition of 4.5 ml of water followed by 10 ml of Insta-Gel 24 hours later.

The results are expressed as disintegrations per minute of ³H-dopamine and ³H-tyrosine per gram of whole rat brain. None of these values is corrected for recovery. Approximate recoveries in percentage: norepinephrine, 90; dopamine, 90; normetanephrine, 89; MOPEG, 58; DOPEG, 27 (Bræstrup et al., 1974).

Tyrosine hydroxylase in crude synaptosomes. The occipital cortex (200 mg) or the striatum (100 mg) was quickly dissected from three rats and homogenized in, respectively, 5 and 10 volumes of 0.32 M sucrose by 4 strokes at 800 rpm in a Potter-Elvehjem glass homogenizer with 0.25 mm clearance. The homogenate was centrifuged at 1000 × g for 10 minutes at 0°C, and the cloudy supernatant containing pinched nerve endings was used. Fifty microliters of synaptosome suspension were added at 0°C to 700 μl of phosphate buffer (NaCl, 122 mM; KCl, 4.8 mM; CaCl₂, 0.97 mM; MgSO₄, 1.21 mM; phosphate, 15.6 mM; EDTA, 0.2 mM; ascorbic acid, 1 mM; glucose, 11 mM; ³H-tyrosine 1.3 × 10⁻⁷ M and the drug to be tested). The buffer was saturated with oxygen just before addition of ascorbic acid, glucose, tyrosine and the test drug. The samples were then incubated at 37°C, pH 7.2, for 1 hour (striatum) or ½ hour (occipital cortex). After incubation, the samples were acidified with 25 μl of 1 M perchloric acid, and, after centrifugation, excess of ³H-tyrosine was removed

from striatal samples by a 1.5 × 0.4 cm Dowex 50X4 column, prewashed with 10 ml of 0.01 M HCl. The column was then washed with 2 × 0.5 ml of 0.01 M HCl. ^{3}H-H$_2$O in the effluent and washings was microdistillated and 100 μl were added to 10 ml of Insta-Gel for tritium estimation.

After acidification with perchloric acid, samples containing occipital cortex synaptosomes were centrifuged and the supernatant was adjusted to pH 5 with 3 ml of 0.25 M acetate buffer. On the next day, ^{3}H-norepinephrine was adsorbed onto 200 mg of alumina at pH 8.4 after addition of 1 ml of 1 M Tris buffer. The alumina was washed with water and ^{3}H-norepinephrine was eluted with 2 × 5 ml of 0.5 M acetic acid containing 10 mg of ascorbic acid and 50 mg of EDTA. ^{3}H-norepinephrine was then purified by adsorption on a Dowex 50X4 column and eluted in 10 ml of 1 M HCl before scintillation counting in 10 ml of Insta-Gel.

Results are expressed in picomoles of ^{3}H-H$_2$O or ^{3}H-norepinephrine per gram of original tissue per hour, corrected for blank value (0°C incubation) and recovery of norepinephrine (80%).

The following drugs and chemicals were used: clonidine, s.c. (gift from Boehringer-Ingelheim, Copenhagen, Denmark; *dl*-propranolol, s.c.; yohimbine, i.p.; apomorphine hydrochloride; α-methyltyrosine methyl ester, i.p., (H 44/68, Hässle), all dissolved in water; phenoxybenzamine and aceperone, i.p. (Janssen Pharmaceuticals) both dissolved in propylene glycol, 1 M HCl 1:1; reserpine, s.c. (Serpasil, gift from Ciba Limited, Copenhagen; ^{3}H-dopamine (3,4-dihydroxyphenylethylamine [ring-^{3}H (G)]-hydrochloride, 500 mCi/mmol, Amersham, England) and ^{3}H-tyrosine (^{3}H-l-tyrosine [ring 3,5-^{3}H] 50 Ci/mmol).

Four to six rats were treated with drugs and a vehicle-treated control group (four–six rats) was always analyzed on the same day to be compared with a maximum of two different drug treatment groups. Student's *t* test of the drug-treated groups *vs.* control groups was used for all statistical evaluations.

Results

Endogenous norepinephrine and dopamine, α-methyltyrosine disappearance.
Clonidine (0.5 mg/kg s.c.), phenoxybenzamine (20 mg/kg i.p.) and aceperone (20 mg/kg i.p.) showed little or no effect on endogenous norepinephrine content in the whole rat brain (table 1).

The disappearance rate of norepinephrine after synthesis inhibition by α-methyltyrosine (H 44/68) was strongly decreased by clonidine (0.5 mg/kg s.c.); the rate constants, estimated by exponential curve fit, were −0.186 hr^{-1} for

norepinephrine after H 44/68 alone and −0.079 hr^{-1} for H 44/68 with clonidine pretreatment (fig. 1). Also, the disappearance of dopamine after H 44/68 was decreased by clonidine (fig. 1). The level of homovanillic acid was reduced in the whole rat brain to 66 ± 7% of control (n = 4; P < .01) 2 hours after 0.5 mg/kg of clonidine s.c. (homovanillic acid control level, 82.2 ± 3.3 ng/g mean ± S.E.M.; n = 4).

Endogenous total MOPEG after clonidine, phenoxybenzamine and aceperone.
Clonidine (0.5 mg/kg) induced a rapid decrease in the level of endogenous total MOPEG. After 1 hour,

TABLE 1

Effects of drugs on the level of endogenous norepinephrine in the rat brain

The means ± S.E.M. of four values are shown as percentage of controls. Control level, 439 ± 27 ng/g (12), corrected for recovery.

Treatment, mg/kg	Time before Decapitation	Norepinephrine
	hr	*% controls*
Clonidine, 0.5	2	121 ± 3[a]
Clonidine, 0.5	4 ½	118 ± 1[a]
Phenoxybenzamine, 20	2	97 ± 2
Phenoxybenzamine, 20	4 ½	92 ± 2
Aceperone, 20	2	87 ± 6
Aceperone, 20	4 ½	103 ± 3

[a] P < .05.
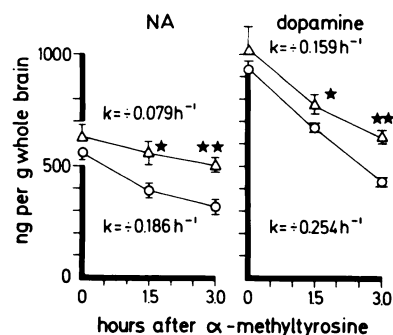

Fig. 1. Clonidine (0.5 mg/kg s.c.) or saline was administered to groups of four rats ½ hour before administration of α-methyltyrosine (H 44/68, 250 mg/kg i.p.), and endogenous norepinephrine (NA) and dopamine were estimated in the whole rat brain at different time intervals after H 44/68. The means ± S.E.M. are shown, all corrected for recovery. O——O, H 44/68 alone; Δ——Δ, clonidine + H 44/68. The rate constants, *k*, were estimated by exponential curve fit. *P < .05; **P < .01 *vs.* H 44/68.

the decrease was evident and maximal effect occurred after approximately 3 hours (fig. 2). After 24 hours, the effects had ceased; in one experiment, a small overshoot in MOPEG was observed (114 ± 2%; P < .01; n = 4).

In contrast to clonidine, phenoxybenzamine (20 mg/kg) and aceperone (20 mg/kg) caused a rapid increase in endogenous total MOPEG. The MOPEG increase induced by aceperone lasted 6 to 8 hours whereas the increase induced by phenoxybenzamine was still present after 24
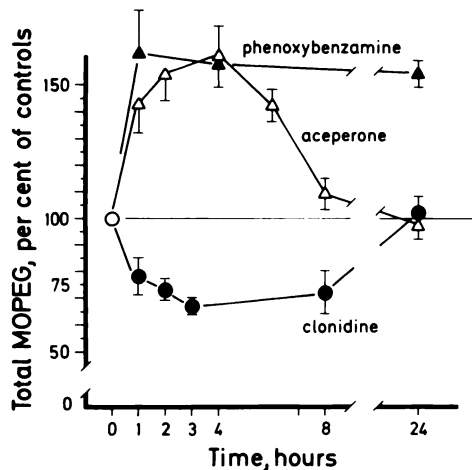
hours (fig. 2). The present finding of increased endogenous MOPEG and unchanged level of norepinephrine indicate that both the utilization and synthesis of norepinephrine were increased by phenoxybenzamine and aceperone.

Decreasing doses of clonidine were tested on endogenous total MOPEG. It appeared that clonidine even at a dose of 0.02 mg/kg (n = 8; P < .001) caused almost maximal decrease in total MOPEG and that no further decrease was observed in the 25-fold dose range from 0.02 to 0.5 mg/kg (fig. 3).

Effect of clonidine on ³H-norepinephrine and its metabolites. Whether ³H-dopamine (table 2) or ³H-tyrosine (table 3) was used as a

FIG. 2. Total endogenous MOPEG in the whole rat brain at various intervals after clonidine (0.5 mg/kg s.c.), aceperone (20 mg/kg i.p.) and phenoxybenzamine (20 mg/kg i.p.). The means ± S.E.M. of four to eight values at each time point are shown. MOPEG control levels, mean ± S.E.M.: for clonidine, 91.3 ± 3.1 ng/g (n = 16); for aceperone 95.1 ± 3.5 ng/g (n = 16); for phenoxybenzamine, 102.8 ± 4.3 (n = 12), all corrected for recovery.
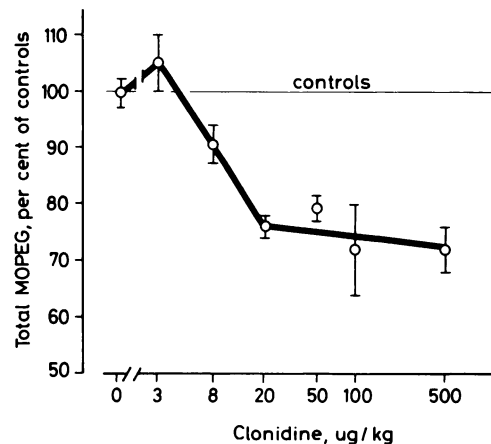

FIG. 3. Total endogenous MOPEG in the whole rat brain 2 hours after increasing doses of clonidine (s.c.). The means ± S.E.M. of four to eight values are shown. Control level, mean ± S.E.M.: 98.5 ± 2.7 ng/g (n = 12), corrected for recovery.

TABLE 2

Effects of clonidine on the accumulation and metabolism of ³H-norepinephrine after intraventricular injection of ³H-dopamine (10 μCi; 1.2 × 10⁷ dpm/g) to rats in vivo

Clonidine (0.5 mg/kg s.c.) was injected ½ hour before ³H-dopamine, and ³H-dopamine was injected ½ or 2 hours before decapitation. The means ± S.E.M. of (n) values are shown.

Pretreatment	³H-Dopamine	³H-Norepineph-rine	³H-MOPEG	³H-DOPEG	³H-Normeta-nephrine	³H-Dopamine
	hr	*dpm/g brain tissue × 10⁻³, mean ± S.E.M.*				
Vehicle	0.5	73 ± 7 (6)	13.2 ± 0.20 (6)	3.93 ± 0.67 (6)	1.40 ± 0.13 (5)	149 ± 23 (6)
Clonidine	0.5	64 ± 7 (6)	9.3 ± 0.14 (6)	1.71 ± 0.22ᵃ (5)	0.89 ± 0.15ᵃ (5)	224 ± 28 (6)
Vehicle	2	44 ± 4 (9)	9.2 ± 0.09 (9)	3.70 ± 0.52 (7)	0.62 ± 0.07 (5)	73 ± 20 (9)
Clonidine	2	66 ± 5ᵇ (9)	5.5 ± 0.09ᵃ (9)	1.90 ± 0.44ᵃ (7)	0.32 ± 0.08ᵃ (5)	157 ± 18ᵃ (9)

ᵃ P < .05.
ᵇ P < .01.

TABLE 3

Effects of clonidine on the accumulation and metabolism of ^{3}H-norepinephrine after intraventricular injection of ^{3}H-tyrosine (50 μCi; 6 × 10^7 dpm/g) to rats in vivo

Clonidine (0.5 mg/kg s.c.) was injected ½ hour before ^{3}H-tyrosine, and ^{3}H-tyrosine was injected ½ or 2 hours before decapitation. The means ± S.E.M. of (n) values are shown.

Pretreatment	^{3}H-tyrosine	^{3}H-Norepineph-rine	^{3}H-MOPEG	^{3}H-DOPEG	^{3}H-Dopamine	^{3}H-Tyrosine
	hr	*dpm/g brain tissue × 10^{-3}, mean ± S.E.M.*				
Vehicle	0.5	34 ± 5 (5)	1.40 ± 0.15 (6)	1.10 ± 0.15 (6)	32 ± 4 (6)	2300 ± 300 (6)
Clonidine	0.5	21 ± 2[a] (6)	0.50 ± 0.09[b] (5)	0.42 ± 0.06[b] (5)	29 ± 3 (6)	3300 ± 400 (6)
Vehicle	2	32 ± 3 (10)	3.47 ± 0.22 (10)	1.86 ± 0.18 (10)	13 ± 2 (9)	490 ± 50 (10)
Clonidine	2	29 ± 3 (9)	1.57 ± 0.24[b] (9)	0.9 ± 0.09[b] (9)	17 ± 3 (9)	560 ± 60 (10)

[a] $P < .05$.
[b] $P < .01$.

precursor, the levels of the two major norepinephrine metabolites ^{3}H-MOPEG and ^{3}H-DOPEG were decreased by pretreatment with clonidine (0.5 mg/kg s.c.), except ½ hour after ^{3}H-dopamine when the ^{3}H-MOPEG decrease was not significant. The accumulation of ^{3}H-norepinephrine was dependent on the precursor and the time interval. Half an hour after ^{3}H-tyrosine injection, clonidine caused a decrease in ^{3}H-norepinephrine accumulation (table 3), whereas an increase was noticed 2 hours after ^{3}H-dopamine (table 2). The minor norepinephrine metabolite ^{3}H-normetanephrine was decreased by clonidine after ^{3}H-dopamine (table 2). These results with norepinephrine accumulation and metabolite formation at two time points strongly indicate that the utilization and also the synthesis of norepinephrine are decreased by clonidine (tables 2 and 3).

^{3}H-normetanephrine cannot be measured with our technique when ^{3}H-tyrosine is used as precursor.

The level of ^{3}H-dopamine appeared to be increased after both precursors. Most of the ^{3}H-dopamine 2 hours after intraventricular tyrosine is present in the striatum (M. Nielsen, unpublished observation), probably without serving as a precursor of ^{3}H-norepinephrine. No significant changes in the level of ^{3}H-tyrosine were observed after clonidine (table 3).

Effects of phenoxybenzamine and aceperone on ^{3}H-norepinephrine and its metabolites. When ^{3}H-dopamine was used a precursor, the levels of the major ^{3}H-norepinephrine metabolites were increased both at ½ hour and at 2 or 3 hours by the two *alpha* adrenoceptor

blocking drugs, phenoxybenzamine (20 mg/kg i.p.) and aceperone (20 mg/kg i.p.) (table 4); only ^{3}H-DOPEG was not increased at ½ hour by phenoxybenzamine. The increased levels of both the major norepinephrine metabolites, in connection with an unchanged or increased norepinephrine accumulation after ½ hour and unchanged or decreased levels of ^{3}H-norepinephrine at 2 to 3 hours, indicate that both phenoxybenzamine and aceperone increase the utilization of ^{3}H-norepinephrine.

When ^{3}H-tyrosine was used as precursor, the level of ^{3}H-MOPEG and ^{3}H-DOPEG was increased at 2 hours but not at ½ hour after ^{3}H-tyrosine injection by phenoxybenzamine and aceperone (table 5). This result indicates increased utilization at least in the time interval from ½ hour to 2 hours. The finding that ^{3}H-tyrosine was increased by phenoxybenzamine and aceperone at ½ hour cannot challenge this conclusion, since even an increased supply of ^{3}H-tyrosine should be expected to result in an equal increase in both ^{3}H-norepinephrine and metabolite accumulation, if utilization was unchanged. We found, however, that only the metabolites were increased (table 5).

No definite conclusions about the synthesis of norepinephrine should be drawn from table 5. The disappearance rate of ^{3}H-tyrosine is extremely high at short time intervals after intraventricular injection, and the high levels of ^{3}H-tyrosine measured ½ hour after injection in the phenoxybenzamine- and aceperone-treated rats may be caused by a small dislocation of the tyrosine disappearance curve. Therefore, it is not reasonable to assume that access of ^{3}H-tyro-

TABLE 4

Effects of phenoxybenzamine (20 mg/kg i.p.) and aceperone (20 mg/kg i.p.) on the accumulation and metabolism of ³H-norepinephrine after intraventricular injection of
³H-dopamine (10 μCi; 1.2 × 10⁷ dpm/g) to rats in vivo

Both drugs were injected ½ hour before ³H-dopamine and the rats were decapitated ½, 2 or 3 hours after ³H-dopamine. The means ± S.E.M. of (n) values are shown.

Pretreatment	³H-Dopamine	³H-Norepineph-rine	³H-MOPEG	³H-DOPEG	³H-Dopamine
	hr	dpm/g brain tissue × 10⁻³, mean ± S.E.M.			
Vehicle	0.5	63 ± 7 (6)	10.1 ± 0.5 (4)	2.9 ± 0.2 (4)	147 ± 25 (5)
Phenoxybenzamine	0.5	71 ± 4 (5)	12.8 ± 0.4ᵃ (4)	3.1 ± 0.1 (4)	181 ± 16 (4)
Aceperone	0.5	72 ± 6 (6)	14.6 ± 1.3ᵇ (5)	4.3 ± 0.3ᵃ (5)	176 ± 29 (6)
Vehicle	2	55 ± 3 (8)	8.6 ± 0.8 (9)	3.0 ± 0.5 (8)	75 ± 10 (9)
Phenoxybenzamine	2	58 ± 2 (8)	13.0 ± 0.5ᵃ (9)	5.2 ± 0.5ᵇ (7)	121 ± 11ᵇ (9)
Vehicle	3	44 ± 3 (9)	5.2 ± 0.3 (8)	1.7 ± 0.3 (9)	51 ± 4 (9)
Aceperone	3	31 ± 2ᵃ (9)	8.3 ± 0.5ᵃ (8)	3.0 ± 0.3ᵃ (8)	40 ± 7 (9)

ᵃ P < .01.
ᵇ P < .05.

TABLE 5

Effects of phenoxybenzamine (20 mg/kg i.p.) and aceperone (20 mg/kg i.p.) on the accumulation and metabolism of ³H-norepinephrine after intraventricular injection of
³H-tyrosine (50 μCi; 6 × 10⁷ dpm/g) to rats in vivo

Both drugs were injected ½ hour before ³H-tyrosine and the rats were decapitated ½ or 2 hours after ³H-tyrosine. The means ± S.E.M. of (n) values are shown.

Pretreatment	³H-Tyrosine	³H-Norepineph-rine	³H-MOPEG	³H-DOPEG	³H-Dopamine	³H-Tyrosine
	hr	dpm/g brain tissue × 10⁻³, mean ± S.E.M.				
Vehicle	0.5	35 ± 1 (16)	3.1 ± 0.3 (16)	2.2 ± 0.23 (15)	40 ± 3 (16)	2500 ± 200 (16)
Phenoxybenzamine	0.5	34 ± 2 (11)	3.0 ± 0.3 (10)	1.87 ± 0.32 (11)	35 ± 4 (11)	3600 ± 400 (11)
Aceperone	0.5	32 ± 1 (11)	2.6 ± 0.3 (11)	1.68 ± 0.20 (11)	42 ± 5 (10)	3300 ± 300 (12)
Vehicle	2	24 ± 1 (23)	3.1 ± 0.2 (23)	2.04 ± 0.16 (23)	13.9 ± 1.4 (22)	377 ± 15 (22)
Phenoxybenzamine	2	23 ± 2 (11)	4.2 ± 0.4ᵃ (11)	3.02 ± 0.36ᵃ (11)	14.0 ± 1.8 (11)	385 ± 24 (11)
Aceperone	2	28 ± 2 (12)	5.2 ± 0.4ᵇ (12)	4.20 ± 0.31ᵇ (12)	22.3 ± 2.3ᵃ (12)	454 ± 21ᵃ (12)

ᵃ P < .05.
ᵇ P < .01.

sine at the site of synthesis in the controls and in the phenoxybenzamine and aceperone groups is equal at short time intervals.

Attempts to antagonize clonidine-induced decrease in endogenous total MOPEG. The results in figures 4 and 5 show different combinations of clonidine and the *alpha* adrenoceptor antagonists, phenoxybenzamine or aceperone. When clonidine was administered before the antagonist, no visible effect of the antago-
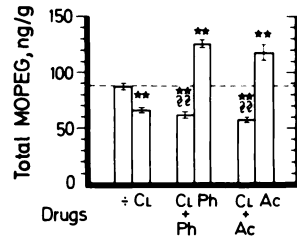
Fig. 4. Inhibition of the phenoxybenzamine (Ph, 20 mg/kg i.p.)- and aceperone (Ac, 20 mg/kg i.p.)-induced increase in total endogenous MOPEG in the whole rat brain by pretreatment with clonidine (Cl, 0.5 mg/kg s.c.). Clonidine was administered 3 hours and phenoxybenzamine and aceperone 2 hours before decapitation. The means ± S.E.M. of four values are shown, all corrected for recovery. **P < .01 vs. control; §§P < .01 vs. antagonist alone.
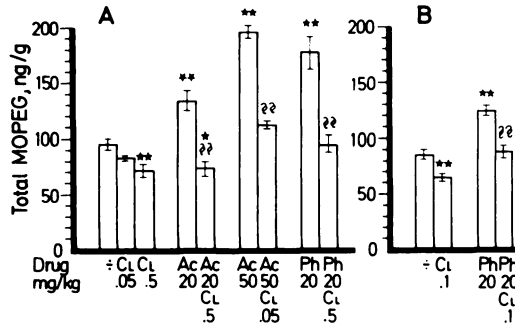

Fig. 5. Failure of phenoxybenzamine (Ph, 20 mg/kg i.p.) and aceperone (Ac, 20 or 50 mg/kg i.p.) to block the effects of subsequent clonidine administration (Cl, 0.05-0.5 mg/kg s.c.) on endogenous total MOPEG in the whole rat brain. The means ± S.E.M. of four values on each treatment are shown, all corrected for recovery. *P < .05; **P < .01 vs. control; §§P < .01 vs. antagonist alone. A. The antagonist was administered 3 hours and clonidine 2 hours before decapitation. B. Phenoxybenzamine was administered 26 hours and clonidine 2 hours before decapitation (see also table 6).

nist was observed (fig. 4). When phenoxybenzamine (20 mg/kg) was administered before clonidine (0.5 mg/kg), the level of MOPEG was not different from controls but was significantly lower than phenoxybenzamine alone. After pretreatment with aceperone (20 mg/kg), clonidine still reduced MOPEG to 78% of no drug treatment (P < .05) (fig. 5A). Phenoxybenzamine, however, only apparently inhibited the action of clonidine in figure 5A, since it is important to consider that clonidine was administered 1 hour after phenoxybenzamine or aceperone, when the level of MOPEG was already increased. Figure 5A further shows that even a high dose of

aceperone did not inhibit a small dose of clonidine and a similar lack of inhibition was observed 26 hours after phenoxybenzamine treatment (fig. 5B).

Table 6 shows that yohimbine, which has *alpha* adrenoceptor blocking activity, strongly increased the level of MOPEG without blocking the effect of clonidine. The *beta* adrenoceptor blocking drug, *dl*-propranolol (20 mg/kg), did not increase the level of endogenous MOPEG (P > .20) and the clonidine effect was unaltered by propranolol (table 6).

Table 7 shows that clonidine can decrease the level of endogenous total MOPEG even after synthesis inhibition with α-methyltyrosine and that the ability of reserpine to increase MOPEG was still present after clonidine pretreatment.

Tyrosine hydroxylation in synaptosomes. The time course of incubation of ^{3}H-tyrosine with crude synaptosomes from rat striatum and occipital cortex is shown in figure 6. ^{3}H-H_2O formation was almost linear for 60 minutes in striatal synaptosomes whereas the ^{3}H-norepinephrine accumulation was almost linear for only 30 minutes in occipital cortex synaptosomes.

Clonidine in a concentration of 10^{-7} to 10^{-4} M did not inhibit ^{3}H-norepinephrine accumula-

TABLE 6

Effects of yohimbine (2 mg/kg i.p.), dl-propranolol (20 mg/kg s.c.) and clonidine (0.1 mg/kg s.c.) on endogenous total MOPEG in the rat brain

Drugs were administered 2 or 3 hours before decapitation. The means ± S.E.M. of (n) values are shown, all corrected for recovery.

Treatment	Time before Decapitation	Endogenous Total MOPEG	Percentage of Vehicle Treatment
	hr	*ng/g*	
Vehicle	3	102.9 ± 4.7 (4)	100
Yohimbine	3	187.5 ± 14.8 (5)[a]	182
Yohimbine + clonidine	3 2	109.9 ± 4.1 (5)[b]	107
Clonidine	2	79.4 ± 1.7 (5)[a]	79
Vehicle	3	86.9 ± 2.8 (8)	100
dl-Propranolol	3	95.8 ± 5.8 (8)	110
dl-Propranolol + clonidine	3 2	70.8 ± 3.7 (8)[a,b]	81
Clonidine	2	71.1 ± 2.4 (8)[a]	82

[a] P < .01 vs. vehicle.
[b] P < .01 vs. antagonist alone.

tion or ³H-H₂O formation (table 8), which indicates that clonidine does not inhibit tyrosine hydroxylation in isolated dopamine or norepinephrine terminals in the rat brain. ³H-norepinephrine accumulation was inhibited by clonidine only at the very high concentration of 10^{-3} M.

Apomorphine (6×10^{-7} M) inhibited tyrosine hydroxylation in both areas.

TABLE 7

Effects of α-methyltyrosine (H 44/68, 100 mg/kg i.p.) and reserpine (7.5 mg/kg s.c.) on the clonidine (0.5 mg/kg s.c.)-induced decrease in endogenous total MOPEG in the rat brain

Drugs were administered 2 to 3 hours before decapitation. The means ± S.E.M. of (n) values are shown, all corrected for recovery.

Treatment	Time before Decapitation	Endogenous Total MCPEG	Percentage of Vehicle Treatment
	hr	*ng/g*	
Vehicle	2¹₂	91.3 ± 3.2 (3)	100
H 44/68	2¹₂	68.1 ± 4.8 (4)	75
H 44/68 + clonidine	2¹₂ / 2	47.3 ± 2.2[a, b] (4)	52
Vehicle	2	116.9 ± 3.1[a] (6)	100
Reserpine	2	151.5 ± 8.4[a] (5)	130
Clonidine	3	72.1 ± 3.8 (5)	62
Clonidine + reserpine	3 / 2	115.0 ± 3.6[b] (5)	98

[a] P < .01 vs. vehicle.
[b] P < .01 vs. H 44/68 and reserpine, respectively.

Discussion

The aim of the present study was to analyze release processes in central norepinephrine neurons *in vivo* by estimation of norepinephrine metabolites. The two major norepinephrine metabolites in the CNS are conjugated 3-methoxy-4-hydroxyphenylglycol (MOPEG) and 3,4-dihydroxyphenylglycol (DOPEG) (Mannarino *et al.*, 1963; Schanberg *et al.*, 1968; Sugden and Eccleston, 1971; Bræstrup, 1973; Stone, 1973; Bræstrup *et al.*, 1974; Ceasar *et al.*, 1974; Nielsen *et al.*, 1974). The level of MOPEG
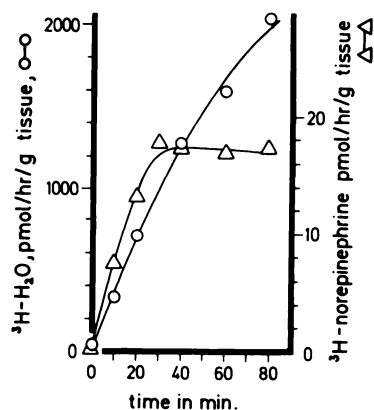
FIG. 6. Accumulation of ³H-H₂O, O——O and ³H-norepinephrine, Δ——Δ, in crude striatal or occipital cortex synaptosomes suspensions, respectively. Synaptosomes were incubated with 1.3×10^{-7} M ³H-tyrosine. Each point is the mean of two determinations.

TABLE 8

Effects of clonidine and apomorphine on tyrosine hydroxylation in striatum and occipital cortex in vitro

Crude synaptosomes from the rat striatum or occipital cortex were incubated with ³H-tyrosine. The formation of ³H-H₂O and ³H-norepinephrine, respectively, is shown as the means ± S.E.M. of N values.

Treatment	N	Drug Conc.	Striatum, ³H-H₂O	Occipital Cortex, ³H-Norepinephrine
		M	*pmol/g/hr original tissue*	
Clonidine	3	0	1635 ± 5	18.5 ± 0.68
	3	10^{-7}	1730 ± 58	18.0 ± 0.51
	3	10^{-6}	1751 ± 21	17.8 ± 0.04
	3	10^{-5}	1677 ± 74	17.5 ± 0.26
	3	10^{-4}	1544 ± 64	16.7 ± 0.44
	3	10^{-3}		7.25 ± 0.25[a]
Apomorphine	4	0	1443 ± 16	18.15 ± 0.4
	4	6.0×10^{-7}	833 ± 11[a]	11.45 ± 0.12[a]
		2.4×10^{-6}		5.22 ± 0.16[a]
0°C, blank	4	0	45 ± 7.4	1.4 ± 0.2

[a] P < .001.

reflects the utilization of norepinephrine and may well be related to release of norepinephrine in the rat brain. When the locus coeruleus is electrically stimulated, the level of endogenous MOPEG is increased in the cortex, a region with norepinephrine nerve terminals (Walter and Eccleston, 1972, 1973; Korf et al., 1973). Further, it has been shown that MOPEG in rat brain is preferentially, although not specifically, formed at extraneuronal sites as should be expected for metabolism of released norepinephrine (Bræstrup and Nielsen, 1975).

The other major norepinephrine metabolite, DOPEG, was incorporated in the study to see whether changes in MOPEG levels were specific to this O-methylated metabolite or whether also DOPEG was affected by *alpha* adrenoceptor active drugs. DOPEG may also be connected to release processes, as it has been indicated that reuptake mechanisms in succession to norepinephrine release will incorporate norepinephrine in the nerve terminals where it may be subject to metabolism *via* monoamine oxidase to DOPEG (Dubocovich and Langer, 1973; Stone, 1973, 1975; Bræstrup and Nielsen, 1975). The results of the present study are compatible with the outlined relation between DOPEG and norepinephrine release.

The most consistent findings of the present study are a decrease in the level of MOPEG and DOPEG after clonidine (tables 2, 3, 6 and 7; figs. 2, 3, 4 and 5) and an increase in the level of MOPEG and DOPEG after phenoxybenzamine and aceperone (tables 4 and 5; figs. 2, 4 and 5). These findings strongly suggest that the release of norepinephrine in the CNS *in vivo* is decreased by clonidine whereas the release is increased by the *alpha* adrenoceptor antagonists aceperone (Janssen et al., 1967) and phenoxybenzamine and also yohimbine (table 6). *In vitro* studies on both central and peripheral tissues have interpreted nerve-stimulated increases and decreases in norepinephrine or norepinephrine metabolite overflow in response to phenoxybenzamine and clonidine as evidence for feedback mechanisms regulating norepinephrine release from nerve terminals (see Introduction). The results of the present study are compatible with the theory of compensatory *alpha* adrenoceptor mediated regulation of norepinephrine release in the CNS *in vivo*. The results, however, do not propagate the notion that phenoxybenzamine and aceperone act on

the same receptors as clonidine or that clonidine has a direct action on the presynaptic noradrenergic terminals (see below).

In vitro studies, especially on hearts where the postsynaptic receptors are of the *beta* type, have indicated a presynaptic target of *alpha* adrenoceptor active drugs (Starke et al., 1971) and studies on cortex slices, where extensive neuronal connections are cut (Farnebo and Hamberger, 1971), indicate that feedback mechanisms can operate locally near synapses. To obtain information on whether clonidine, phenoxybenzamine and aceperone acted on the same receptors, we investigated their mutual antagonism. It appeared that clonidine efficiently blocked the effect of aceperone or phenoxybenzamine (fig. 4) in agreement with *in vitro* studies (Starke and Montel, 1973). On the contrary, it was not possible to block the effect of even 0.05 to 0.1 mg/kg of clonidine by pretreatment with either aceperone (up to 50 mg/kg) or by phenoxybenzamine (20 mg/kg) after 26 hours when nonspecific effects have ceased but when irreversible blockade of the *alpha* adrenoceptor is still present. The drug yohimbine, which reduced the clonidine-induced depression in avoidance conditioned reflexes (Delbarre and Schmitt, 1974) and which blocked the effect of clonidine in the α-methyltyrosine test (Andén and Grabowska, 1975), did not block the clonidine-induced decrease in endogenous MOPEG, although alone it increased total endogenous MOPEG as did the other *alpha* adrenoceptor antagonists. The failure to block clonidine by *alpha* adrenoceptor antagonists may indicate that clonidine and phenoxybenzamine, aceperone or yohimbine do not act on the same receptor and that the site of action of clonidine may be situated earlier in the chain of events leading to release than that of *alpha* adrenoceptor antagonists.

Recently, it was shown that the apomorphine-induced decrease in tyrosine hydroxylase in striatal synaptosomes could be reduced by dopamine receptor antagonists (Ebstein et al., 1974; Christiansen and Squires, 1974; Iversen et al.). This result was interpreted as presynaptic-induced feedback inhibition by the dopamine receptor agonist, apomorphine. In the present study, we failed to show a similar clonidine-induced inhibition of norepinephrine synthesis in crude synaptosomes from occipital cortex, an

area with norepinephrine terminals and apparently no dopamine terminals (Squires, 1974), and as expected we also failed to reduce synaptosomal tyrosine hydroxylase in the dopamine-rich area, striatum. Provided that presynaptic receptors may operate in synaptosomes *in vitro*, the result in table 5, showing that clonidine in concentrations lower than 10^{-4} M is inactive on synaptosome catecholamine formation, further indicates that this drug may act earlier than on hypothesized presynaptic receptors in norepinephrine terminals.

Pertinent to this early action of clonidine is the concept of inhibitory autoreceptors on cell bodies (Carlsson, 1975; Bunney and Aghajanian, 1975) as well as the indication of inhibitory adrenaline receptors on the norepinephrine cell bodies of locus coeruleus (Bolme et al., 1974). Furthermore, Svensson et al. (1975) showed that the firing rate in noradrenergic neurons in the locus coeruelus was inhibited by direct application of clonidine, and it is therefore a likely possibility that the decreased norepinephrine release induced by clonidine is mediated by a direct inhibitory action on noradrenergic cell bodies.

The data in tables 2, 3 and 7 supply an interesting connection between changes in synthesis and changes in release of norepinephrine. It appears that the decrease in norepinephrine synthesis and turnover induced by clonidine and maybe also the increase induced by phenoxybenzamine are secondary to changes in norepinephrine release. The disappearance rate of norepinephrine is reduced by clonidine, even after synthesis inhibition by α-methyltyrosine (Andén et al., 1970; Andén and Grabowska, 1975; fig. 1), and clonidine still possesses its decreasing effect on endogenous MOPEG after inhibition of tyrosine hydroxylase by α-methyltyrosine (table 7). When ^{3}H-tyrosine is used as precursor, clonidine decreases the synthesis of norepinephrine *in vivo* (Rochette and Bralet, 1975; present study), whereas synthesis is increased after phenoxybenzamine and aceperone (Dairman et al., 1968; Persson, 1970; Meek and Neff, 1973; present study). Yet when ^{3}H-DA is used as precursor, thus bypassing the tyrosine hydroxylase enzyme, there is no evidence of decreased or increased synthesis of norepinephrine after clonidine and *alpha* adrenoceptor antagonists, respectively (on the contrary, an increase in the accumulation of ^{3}H-norepineph-

rine was observed after clonidine), and even then the level of MOPEG and DOPEG was decreased after clonidine and increased after phenoxybenzamine or aceperone. This finding substantiates the observation that changes in norepinephrine release induced by *alpha* adrenoceptor active drugs are not necessarily dependent on changes in tyrosine hydroxylase.

Apomorphine has a biochemical effect on the brain dopamine synthesis *in vivo* which is similar to that of clonidine on the norepinephrine system (Roos, 1969; Nybäck et al., 1970; Kehr et al., 1972), and the data on apomorphine are believed to show feedback inhibition of dopamine release by the dopamine receptor agonist. Recently it was suggested that the decreased level of metabolites after apomorphine was due to inhibition of dopamine deamination (DiChiara et al., 1974) rather than to a decreased release. No indication of a similar mechanism of action of clonidine was observed in the present study. The decrease observed in ^{3}H-normetanephrine after clonidine (table 2) shows that clonidine does not merely inhibit norepinephrine metabolism by inhibition of deamination, because the methylated, non-deaminated metabolite (normetanephrine) then would be expected to accumulate. Moreover, the drug reserpine, which exposes stored norepinephrine to metabolism, is still able to produce an increase in the level of MOPEG after clonidine pretreatment although the absolute increase is reduced (table 7), thereby indicating intact deamination properties.

The *beta* adrenoceptor blocking drug propranolol did not affect endogenous total MOPEG, nor was the effect of clonidine antagonized. This finding agrees with *in vitro* results on cortex slices, showing that electrically stimulated overflow of norepinephrine is not dependent on *beta* adrenoceptor activity (Farnebo and Hamberger, 1974).

Increased levels of norepinephrine metabolites after phenoxybenzamine *in vitro* have been interpreted as a reserpine-like effect (Adler-Graschinsky, 1972; Graefe et al., 1973). Phenoxybenzamine increases norepinephrine metabolites *in vivo* (present study; Meek and Neff, 1973; Bræstrup, 1974), as does reserpine (Bræstrup and Nielsen, 1975; present study). In contrast to phenoxybenzamine, however, reserpine depletes norepinephrine stores and inhibits norepinephrine synthesis (Bræstrup and Niel-

sen, 1975) whereas phenoxybenzamine does not decrease norepinephrine synthesis. The effects of phenoxybenzamine observed in the present study are therefore more reasonably ascribed to its *alpha* adrenoceptor antagonistic activity (see also Bræstrup, 1974).

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