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RELEASE OF ENDOGENOUS CATECHOLAMINES FROM ISOLATED RAT BRAIN TISSUE BY FENFLURAMINE AND *N*-ETHYLAMPHETAMINE—EFFECTS OF PARGYLINE*

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Abstract—The influence of the monoamine oxidase inhibitor, pargyline, upon the release of endogenous norepinephrine from chopped rat cerebral cortex and endogenous dopamine from chopped rat corpus striatum by fenfluramine and *N*-ethylamphetamine was examined. Endogenous norepinephrine and dopamine were measured using an enzymatic–isotopic assay. Both fenfluramine and *N*-ethylamphetamine released significant amounts of each catecholamine. Fenfluramine-induced catecholamine release was associated with the decrease in the total content (i.e. catecholamine in media + catecholamine in tissue) of both cortical norepinephrine and striatal dopamine; *N*-ethylamphetamine decreased only total striatal dopamine content. In brain tissue obtained from rats pretreated with pargyline HCl (100 mg/kg, 12 hr prior to sacrifice), total cortical norepinephrine content was approximately twice that in the absence of pargyline; striatal dopamine was unchanged. Pargyline pretreatment also resulted in a marked potentiation of the amounts of both catecholamines released by each drug, and in an antagonism of the above drug-induced reductions in catecholamine content. Additionally, after pargyline pretreatment, *N*-ethylamphetamine reduced total cortical norepinephrine content. When pargyline (2.56×10^{-4} M) was added to the media containing chopped cortical tissue from unpretreated animals, control content was slightly increased. Only fenfluramine-induced norepinephrine release was potentiated and the degree of potentiation was less than that observed after pargyline pretreatment. As with pargyline *in vivo*, pargyline *in vitro* also resulted in an antagonism of the fenfluramine-induced decrease in total norepinephrine and was associated with an *N*-ethylamphetamine-induced decrease in norepinephrine content. Since the locomotor stimulant effects of both fenfluramine and *N*-ethylamphetamine are potentiated after pargyline, the data are consistent with the importance of catecholamines to these effects. The data also suggest that pargyline potentiates the behavioral effects of fenfluramine and *N*-ethylamphetamine in part by increasing the pool of norepinephrine available for release by these drugs and in part by inhibiting the deamination of the released norepinephrine and dopamine by monoamine oxidase. Decreases in total norepinephrine produced by *N*-ethylamphetamine may reflect alterations in the formation of *O*-methylated amines.

N-ethylamphetamine (NEA), like amphetamine [1, 2], is a stimulant of locomotor activity in rodents [3], while fenfluramine (CFEA), the *meta*-trifluoromethylated derivative of NEA, has little if any stimulant effect [3, 4]. When animals are pretreated with the monoamine oxidase inhibitor, pargyline, however, CFEA is an effective locomotor stimulant [3, 4] and the NEA locomotor activity dose–response curve is shifted to the left [3].

The stimulant actions of amphetamine and its congeners have been attributed to the release of the central catecholamines, norepinephrine and dopamine [5, 6]. Consequently, potentiation of the locomotor stimulant actions of NEA and CFEA by pargyline suggests that deamination of catecholamines by monoamine oxidase occurs during release of the amines. Blockade of this metabolic pathway, therefore, would allow more of the released catecholamines to escape degradation, thus increasing the synaptic catecholamine concentration. Indeed, Ziance and Rutledge [7], and Tessel and Rutledge [8] demonstrated that CFEA released deaminated metabolites of accumulated exogenous [^{3}H]catecholamines. It was also found that addition *in vitro* of pargyline resulted in potentiation of the releasing effect of CFEA. However, the release produced by NEA of [^{3}H]norepinephrine from rat cerebral cortex or [^{3}H]dopamine from corpus striatum is not associated with increases or decreases in ^{3}H-deaminated metabolites [8].

Another explanation for the potentiating effects of pargyline pretreatment related to increases in whole brain catecholamine content produced *in vivo* by pargyline administration [9, 10]. Pargyline pretreatment, therefore, would potentiate the locomotor stimulant actions of NEA and CFEA by increasing the amount of catecholamines available for release by these drugs. The purpose of the

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present study has been to test this latter hypothesis by comparing NEA and CFEA with regard to the potency and efficacy with which each drug released endogenous catecholamines from isolated rat brain tissue.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–200 g) were decapitated, and the brains were removed and dissected according to the procedure described by Glowinski and Iversen [11]. The tissue was weighed and chopped into 0.3-mm sections using a mechanical tissue chopper [12].

Release of exogenous norepinephrine and dopamine from chopped rat brain tissue. The tissue was suspended in 2.0 ml of physiological salt solution [13], equilibrated with 95% O_2–5% CO_2, and centrifuged for 2 min at 1000 *g* at room temperature (25°) in an IEC clinical centrifuge. The resulting pellet was resuspended in 3.0 ml of physiological salt solution and 0.5-ml aliquots (approximately 50 mg tissue) of the suspension were added to polyethylene centrifuge tubes. The tubes were centrifuged as described above, the supernatant fractions were discarded, and the tissue pellets resuspended in 1.0 ml of physiological salt solution to which the appropriate drug had been added. Unless otherwise specified, the samples were incubated for 30 min at 37° in a shaking water bath at a speed of 288 agitations/min. After this incubation, the medium was separated from the tissue by centrifugation as described above. The medium was decanted into a second set of tubes containing 2.0 ml of glass distilled water and 25 μl of concentrated (70–72%) perchloric acid. The tissue pellets were homogenized in 3.0 ml of 0.1 N perchloric acid using a glass homogenizer. The samples were centrifuged for 10 min at 12,000 *g* at 4° in a Sorvall RC2-B refrigerated centrifuge. The supernatant fractions were decanted and the pellets were saved for protein determinations [14].

Measurement of endogenous norepinephrine and dopamine. The assay for norepinephrine and dopamine was based on a modification [15] of the procedure described by Coyle and Henry [16]. Perchloric acid was added to samples of medium, to a final concentration of 0.1 N. Three aliquots (300 μl) of each sample were assayed; 10 ng norepinephrine and 5 ng dopamine were added to two of the aliquots to serve as internal standards for the assay. A reaction mixture was prepared and 100 μl of this mixture was added to each sample to begin the enzymatic *O*-methylation. The final concentrations or amounts of the reactants in each sample were as follows: 8.0 mM dithiothreitol; 1.25 mM $MgCl_2$; 0.35 M Tris buffer, pH 9.6; 0.5 μCi [^{3}H]*S*-adenosylmethionine ([^{3}H]SAM) (6.8 to 12.6 Ci/m-mole); and catechol-*O*-methyltransferase, 20 μl of standardized enzyme. The samples were incubated for 60 min at 37°. At the end of the incubation, the reaction was terminated by the addition of 200-μl aliquots of 0.75 M sodium borate containing 34.4 nmoles of 3-methoxytyramine, 13.7 nmoles normetanephrine and 12.8 nmoles metanephrine. The [^{3}H]-*O*-methylated products of the reaction were extracted into 4.5 ml of toluene–isoamyl alcohol (3:2, v/v). The extraction was repeated and the combined organic phase was washed with 500 μl of 0.5 M sodium borate, pH 10. The [^{3}H]-*O*-methylated amines were then extracted into 500 μl of 0.1 N HCl which was washed with 6.0 ml of toluene–isoamyl alcohol (3:2). The separation of [^{3}H]normetanephrine from [^{3}H]3-methoxytyramine was accomplished by subjecting the metabolites to periodate cleavage. The acidic aqueous phase resulting from the previous extraction was made alkaline by the addition of 50 μl of 2.0 N NaOH. Periodate oxidation was initiated by adding 25 μl of 3% $NaIO_4$ to each sample and incubating for 6 min at 20°. The reaction was terminated by the addition of 25 μl of 10% $Na_2S_2O_5$. The samples were acidified with 4.0 N HCl (50 μl each), and [^{3}H]methylvanillin ([^{3}H-methyl]3-methoxy-4-hydroxybenzaldehyde) was extracted into 6.0 ml toluene. The organic phase was decanted into a vial containing 0.6 ml of a mixture containing 50 g of 2,5-diphenyloxazole/liter of methanol. The radioactivity in the aqueous phase, which contained [^{3}H]methoxytyramine formed from dopamine, was determined in vials containing 6.0 ml of toluene–Triton X-100 (2:1, v/v) and 4.0 g of 2,5-diphenyloxazole/liter. The assay was linear from 0 to 20 ng of either norepinephrine or dopamine. In typical samples taken from release experiments, the amounts of norepinephrine and dopamine were in the 1–10 ng range. In samples prepared from cerebral cortex, crossover (the percentage of DA appearing in the NE fraction) was always less than 10 per cent. Values for ng amine/g of tissue were always corrected for crossover. In samples derived from corpus striatum, corrections for crossover of NE into the DA fraction were not made, and these samples not subjected to periodate cleavage since the corpus striatum contains very little NE [17]. Purification of rat liver catechol-*O*-methyltransferase (COMT) was carried out according to the method of Nikodejevic *et al.* [18].

Substances used. d,l-Fenfluramine HCl was obtained from the A. H. Robbins Co., Richmond, VA. *d,l*-Ethylamphetamine HCl was synthesized as previously described [3]. [^{3}H]*S*-adenosylmethionine (6.8 to 12.6 Ci/m-mole) was obtained from Amersham/ Searle Corp., Arlington Heights, IL. Norepinephrine hydrochloride (*d,l*-arterenol hydrochloride), dopamine hydrochloride (3-hydroxytyramine hydrochloride), 3-methoxytyramine, metanephrine and normetanephrine were obtained from CalBiochem, Los Angeles, CA.

Data analysis. The EC_{50} for release was estimated as the concentration of drug which gave half the maximal response. The significance of differences between means was assessed by the use of Student's *t*-test.

RESULTS

Release of endogenous norepinephrine from chopped rat cerebral cortex. Incubation of fenfluramine with chopped cerebral cortex resulted in a concentration-dependent increase in the amount of norepinephrine released into the incubation medium; the amount released was significantly greater than the control at all concentrations tested

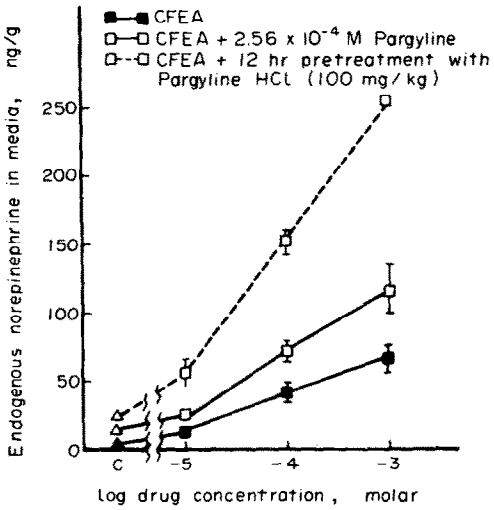

Fig. 1. Release of endogenous norepinephrine from chopped rat cerebral cortex by fenfluramine (CFEA). Release is expressed as ng of amine in the medium/g of tissue. The tissue was incubated for 30 min in physiological salt solution either in the absence (C) or presence of various concentrations of *d,l*-fenfluramine and either in the presence or absence of pargyline. The amount of norepinephrine in the tissue and incubation medium was determined by an enzymatic–isotopic assay. Each value in the presence of fenfluramine represents the mean ± S.E.M. of three to six observations; values associated with C represent the mean ± S.E.M. of twelve observations.

($P < 0.01$; Fig. 1). In the presence of 2.56×10^{-4} M pargyline, the amount of norepinephrine released in the absence of fenfluramine was increased approximately 2.6-fold ($P < 0.01$). However, 10^{-4} M and 10^{-3} M fenfluramine released significantly more of the neurotransmitter in the presence of pargyline than in its absence even when differences in control release were accounted for by subtracting the appropriate control release from each of the fenfluramine-associated points ($P < 0.01$).

When brain tissue was obtained from animals pretreated with pargyline (100 mg/kg of pargyline, 12 hr prior to sacrifice), release of norepinephrine in the absence of drug was nearly twice that observed in experiments in which pargyline was added *in vitro* ($P < 0.01$) and fenfluramine (10^{-4} M and 10^{-3} M) released significantly more neurotransmitter after *in vivo* than after *in vitro* pargyline ($P < 0.01$).

N-ethylamphetamine also released endogenous norepinephrine (Fig. 2), each concentration tested releasing significantly more than was released in the absence of drug ($P < 0.05$ for 10^{-6} M; $P < 0.01$ for 10^{-5} M to 10^{-3} M). Although release in the absence of drug was approximately 3-fold greater in the presence of 2.56×10^{-4} M pargyline, *N*-ethylamphetamine-induced release of norepinephrine was not markedly altered compared to that in the absence of pargyline. In contrast, the amount released by each concentration of *N*-ethylamphetamine was potentiated after *in vivo* pargyline ($P < 0.01$).

Figure 3 illustrates the effects of both fenfluramine and pargyline on total norepinephrine content (tissue + medium). In the absence of pargyline, fenfluramine produced a concentration-related de-

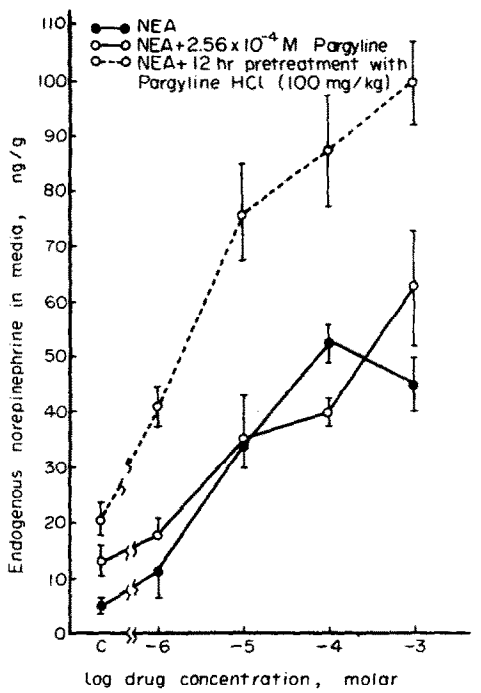

Fig. 2. Release of endogenous norepinephrine from chopped rat cerebral cortex by *N*-ethylamphetamine (NEA). Release is expressed as ng amine in the medium/g of tissue. The tissue was incubated for 30 min in physiological salt solutions either in the absence (C) or presence of various concentrations of *d,l-N*-ethylamphetamine, and either in the presence or absence of pargyline. The amount of norepinephrine in the tissue and incubation medium was determined by an enzymatic–isotopic assay. Each value in the presence of *N*-ethylamphetamine represents the mean ± S.E.M. of three to six observations; values associated with C represent the mean ± S.E.M. of twelve observations.

crease in content that was significant at both 10^{-4} M and 10^{-3} M ($P < 0.01$). Addition *in vitro* of pargyline was associated with a small but significant increase in content ($P < 0.01$) and completely

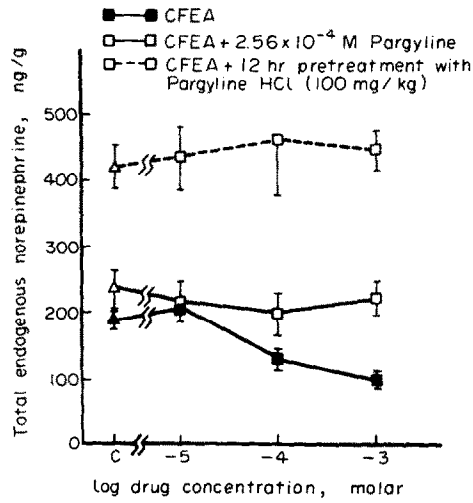

Fig. 3. Total endogenous norepinephrine (i.e. tissue + media) in chopped rat cerebral cortex. See Fig. 1 for details.

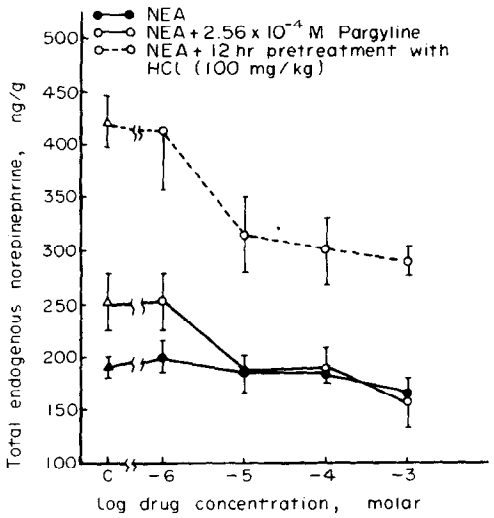

Fig. 4. Total endogenous norepinephrine (i.e. tissue + media) in chopped rat cerebral cortex. See Fig. 2 for details.

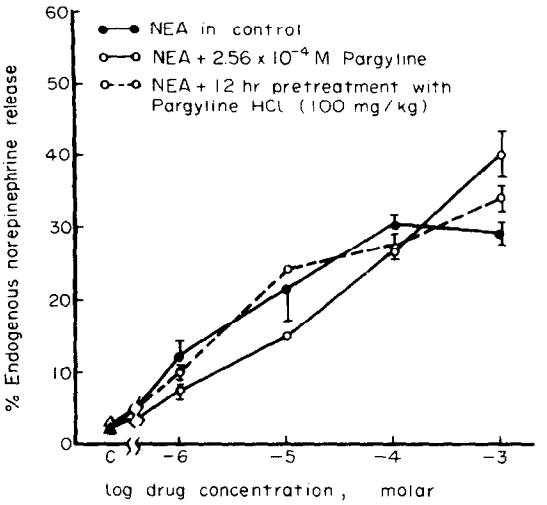

Fig. 6. Percentage of total endogenous norepinephrine in chopped rat cerebral cortex found in the media. See Fig. 2 for details.

blocked the decrease in norepinephrine produced by fenfluramine. In animals pretreated with pargyline, norepinephrine content was approximately doubled, and, as with pargyline administered *in vitro*, the decrease in the content of fenfluramine produced was abolished.

In the absence of pargyline, *N*-ethylamphetamine (in contrast to fenfluramine) did not alter control norepinephrine content at any concentration (Fig. 4). However, in the presence of pargyline, regardless of the mode of pargyline administration, norepinephrine content was significantly decreased by higher concentrations of the drug ($P < 0.01$).

Because of the differences between the effects of the two drugs on norepinephrine content and release after pargyline, the relative potency and efficacy of the two drugs cannot be readily compared. This difficulty can be circumvented to some extent by comparing the percentages of total norepinephrine (media + tissue) that were found in the media. These data are illustrated in Figs. 5 and 6. *N*-ethylamphetamine was more potent and significantly less efficacious ($P < 0.01$) than fenfluramine in releasing norepinephrine regardless of the presence

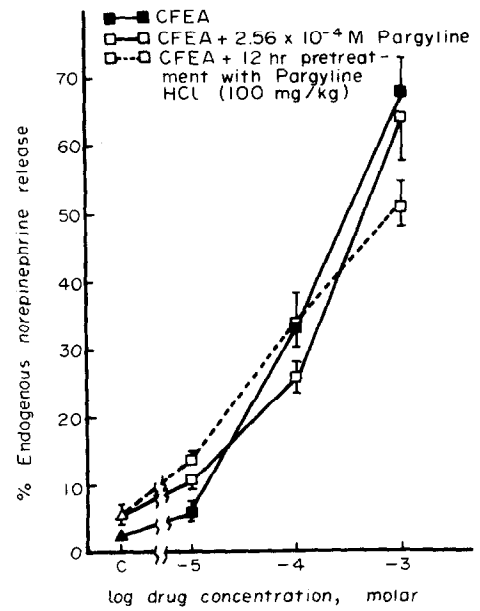

Fig. 5. Percentage of total endogenous norepinephrine in chopped rat cerebral cortex. See Fig. 1 for details.

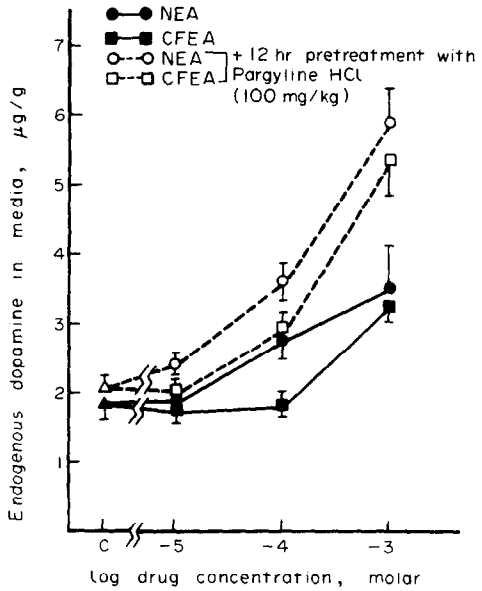

Fig. 7. Release of endogenous dopamine from chopped rat corpus striatum. Release is expressed as μg amine in the medium/g of tissue. The tissue was incubated for 30 min in physiological salt solutions either in the absence (C) or presence of various concentrations of *d,l*-*N*-ethylamphetamine (NEA) or *d,l*-fenfluramine (CFEA) and either in the presence or absence of pargyline pretreatment. The amount of dopamine in the tissue and incubation medium was determined by an enzymatic-isotopic assay. Each value in the presence of drug represents the mean ± S.E.M. of three to five observations; values associated with C represent the mean ± S.E.M. of twelve observations.

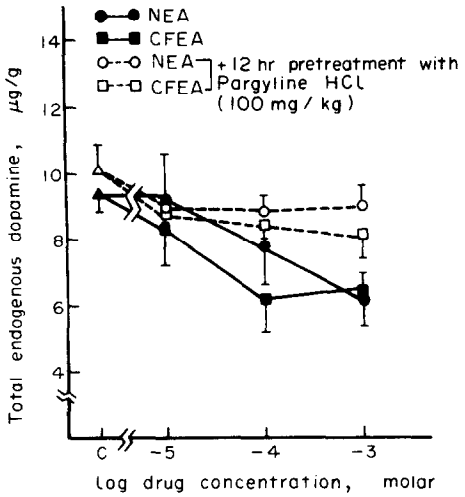

Fig. 8. Total endogenous dopamine (i.e. tissue + media) in chopped rat corpus striatum. See Fig. 7 for details.

of pargyline. Pargyline did not alter the potency with which fenfluramine released norepinephrine, the EC_{50} value being approximately 1.2×10^{-4} M. Only pargyline *in vitro* changed the potency with which *N*-ethylamphetamine released norepinephrine. The EC_{50} values were as follows: in the absence of pargyline, 4.6×10^{-6} M; in the presence of pargyline *in vitro*, 4.3×10^{-5} M; and after pargyline *in vivo*, 5.2×10^{-6} M. Pargyline had little effect upon the maximal percentages of norepinephrine released by either drug.

Release of endogenous dopamine from chopped rat corpus striatum. Both drugs increased the amount of dopamine in the incubation medium (Fig. 7). However, only high concentrations of each drug released significant amounts of dopamine ($P < 0.01$). The amount of dopamine released by both drugs from corpus striatum was increased after pargyline pretreatment ($P < 0.01$) even though control release was not significantly altered.

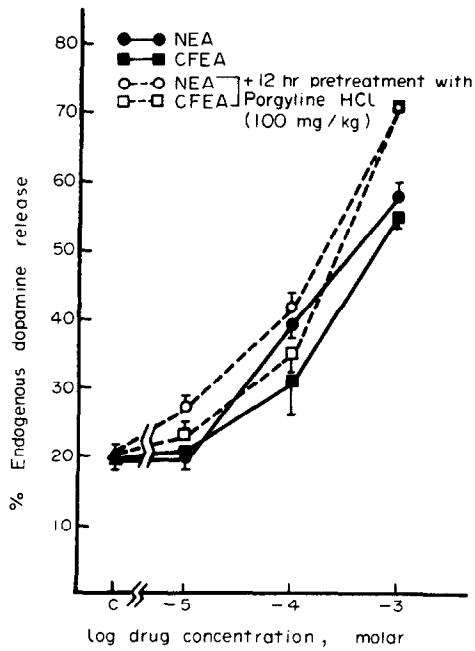

Fig. 9. Percentage of total endogenous dopamine in chopped rat cerebral cortex found in the media. See Fig. 7 for details.

As with cerebral norepinephrine content, fenfluramine (10^{-4} M and 10^{-3} M) decreased striatal dopamine content (Fig. 8; $P < 0.01$). *N*-ethylamphetamine also decreased dopamine content, in contrast to its effect on norepinephrine content, but only at 10^{-3} M ($P < 0.01$). Pargyline pretreatment abolished the decrease in content produced by *N*-ethylamphetamine and antagonized that due to fenfluramine, but did not significantly alter dopamine content.

On a percentage basis, both drugs were relatively similar in potency (EC_{50} for fenfluramine = 2.9×10^{-4} M and EC_{50} for *N*-ethylamphetamine = 8.8×10^{-5} M), each releasing approximately 56 per cent at 10^{-3} M (Fig. 9). Pargyline pretreatment did not alter the percentage of dopamine released in the absence of drug (approximately 19 per cent in both the absence and presence of pargyline) or markedly change the percentage released by either drug; the potencies with which fenfluramine and *N*-ethylamphetamine released dopamine were 2.0×10^{-4} M and 1.2×10^{-4} M respectively.

DISCUSSION

The locomotor stimulant actions of *N*-ethylamphetamine are potentiated in animals pretreated with the monoamine oxidase inhibitor, pargyline [3, 4]. Although the mechanisms by which these drugs affect locomotor activity are not clear, they appear to involve the release of central catecholamines [3, 8]. Presumably, then, the ability of pargyline to potentiate these locomotor stimulant actions of the above drugs is related to catecholamine release. In the present study, pargyline both *in vitro* and *in vivo* potentiated fenfluramine-induced release of endogenous cortical norepinephrine and antagonized the decreases both in norepinephrine and in endogenous striatal dopamine produced by fenfluramine. These data, taken together with those for exogenously administered [^{3}H]catecholamines [7, 8], indicate that to a large extent fenfluramine releases deaminated metabolites of catecholamines. When deamination is blocked by pargyline, more of the released catecholamines reach the synapse in a physiologically active form. In addition, pargyline pretreatment increased the cerebral content of norepinephrine. Since fenfluramine released significantly more norepinephrine from cortical tissue obtained from animals pretreated with pargyline than from tissues treated *in vitro* with pargyline, the size of the pool of norepinephrine available for release is probably involved in the pargyline-induced potentiation of fenfluramine's actions on locomotor activity. That this pool is the same as that involved in the absence of pargyline is suggested by the finding that the percentage of total norepinephrine which is released is relatively unchanged by pargyline.

N-ethylamphetamine decreased norepinephrine content only in the presence of pargyline regardless

of the mode of pargyline administration. This latter finding has several possible interpretations. One possibility could be that *N*-ethylamphetamine inhibited the rat liver catechol-*O*-methyltransferase used in the assay for endogenous norepinephrine and dopamine. However, if this had been the case, increasing concentrations of *N*-ethylamphetamine should have also been associated with a decrease in endogenous norepinephrine content in the absence as well as in the presence of pargyline. Additionally, *d*-amphetamine does not inhibit this enzyme under conditions similar to those in the present study (unpublished data).

NEA in combination with pargyline could also result in inhibition of norepinephrine synthesis with the added possibility of the released norepinephrine being metabolized by catechol-*O*-methyltransferase. Such an increase in the amount of *O*-methylated metabolites might occur because the only major alternative for norepinephrine metabolism is inhibited by pargyline; *O*-methylated metabolites would not be detected with the assay procedure used here. Although little direct evidence exists for inhibition of tyrosine hydroxylase in central noradrenergic neurons by *N*-ethylamphetamine or even amphetamine, amphetamine does appear to inhibit dopamine-β-hydroxylase [19] in the central nervous system. Additionally, monoamine oxidase inhibitors administered *in vivo* [20, 21] have been shown to inhibit the formation of norepinephrine from tyrosine in central and peripheral noradrenergically innervated tissue, and both pargyline and amphetamine inhibit *in vitro* tyrosine hydroxylase in intact peripheral tissue [22]. These latter effects are presumed to be due to an increase in free intraneuronal norepinephrine which competes for the reduced pteridine cofactor for tyrosine hydroxylase [22].

N-ethylamphetamine was approximately 30-fold more potent than fenfluramine in releasing endogenous norepinephrine. In addition, release of norepinephrine by *N*-ethylamphetamine reached a plateau between 10^{-4} M and 10^{-3} M, while release associated with fenfluramine did not. Similar data were obtained by Tessel and Rutledge [8] concerning the release of newly accumulated [^{3}H]norepinephrine from rat cerebral cortex. It was demonstrated that the differences in potency between these two drugs may be attributed to the size of the trifluoromethyl group in the fenfluramine molecule which apparently limits the access of the drug to the intraneuronal compartment. The data of Tessel and Rutledge [8] suggest that *N*-ethylamphetamine enters the noradrenergic neuronal ending at a faster rate, at least in part, by utilizing the neuronal neurotransmitter uptake system to gain entry into the cell. However, at high concentrations the uptake system becomes saturated and consequently norepinephrine release becomes limited because drug entrance into the neuron is limited. Fenfluramine, because of its large bulk and high lipophilicity [23], probably depends primarily upon simple diffusion to gain entry into the noradrenergic neuron.

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