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ASPECTS OF THE BIOGENIC AMINE-DEPLETING ACTIVITY OF ETHYL 3-ACETAMIDO-4H-PYRROLO-[3,4-c]ISOXAZOLE-5(6H)-CARBOXYLATE IN THE RAT

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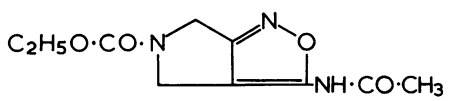
Agents have been found which cause marked lowering of the catecholamine and/or 5-hydroxytryptamine concentrations in tissues and some (for example, α-methyl-dihydroxyphenylalanine, guanethidine and reserpine) are useful in the treatment of hypertension (Hafkenschiel & Sellers, 1954 ; Richardson & Wyso, 1959 ; Hollander, 1961 ; Page, Hurley & Dustan, 1961 ; Gillespie, Oates, Crout & Sjoerdsma, 1962 ; Cannon, Whitlock, Morris, Angers & Laragh, 1962). Ethyl 3-acetamido-4H-pyrrolo[3,4-*c*]-isoxazole-5(6H)-carboxylate (CL-62375) has been reported to decrease both the catecholamine and 5-hydroxytryptamine concentrations in tissues of the rat (Lippmann & Wishnick, 1967). After a single administration of CL-62375 there is an appreciable lowering of the catecholamine concentration in the heart and brain and a slight fall in brain 5-hydroxytryptamine. After repeated administration of CL-62375 the heart shows the largest decrease in the catecholamine concentration followed by the brain and the adrenals ; the brain 5-hydroxytryptamine decreases appreciably. CL-62375 causes a lowering of blood pressure in the rat (Cummings & Welter, 1967, personal communication). Studies on the mechanism of the biogenic amine-depleting activity of CL-62375 were carried out and the results are presented in the present paper.



Ethyl 3-acetamido-4H-pyrrolo[3,4-c]isoxazole-5(6H)carboxylate

METHODS

Catecholamine and 5-hydroxytryptamine concentrations in tissues

Catecholamine concentrations in the brain were determined as described by Lippmann & Wishnick (1965) essentially according to the procedure of Maynert & Klingman (1962), a modification of the Shore & Olin method (1958) in which ferricyanide was substituted for iodine (von Euler & Floding, 1955), and noradrenaline was used as the standard. The amines were extracted from aqueous acidic homogenates of brain into butanol, extracted back into an acidic aqueous phase and the noradrenaline

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was converted to a fluorescent derivative by oxidation with ferricyanide. 5-Hydroxytryptamine concentrations in the brain were assayed by the fluorometric procedure of Bogdanski, Pletscher, Brodie & Udenfriend (1956) on aliquots of the final acid extract (Mead & Finger, 1961), by determination of the amount of fluorescence, measured at a wavelength of 550 mμ, of the sample in 3N HCl. Heart noradrenaline was isolated and assayed as described by Whitby, Axelrod & Weil-Malherbe (1961) and involved the selective adsorption of the catecholamine on to aluminium oxide and the oxidation of the noradrenaline in the subsequent acetic acid eluate to form a fluorescent derivative. Adrenal catecholamines were isolated and determined as previously described (Lippmann & Wishnick, 1965) and this consisted of an extraction of the catecholamines into an acidic solution and a subsequent oxidation to give a fluorescent product.

Radioactive tyrosine and noradrenaline concentrations in tissues

[^{3}H]-Tyrosine was measured by liquid scintillation counting in Bray's solution (Bray, 1960) on aliquots of effluents from alumina columns (Udenfriend & Zaltzman-Nirenberg, 1963). [^{3}H]-Noradrenaline was measured by liquid scintillation counting in Bray's solution on aliquots of a 0.2 N acetic acid eluate from alumina columns (Whitby, Axelrod & Weil-Malherbe, 1961).

Drug administration

Rats used were female albinos of the Sherman strain from Wyckoff Farms colony (average weight, 150 g). 3,5-[^{3}H]-*l*-Tyrosine (4.0 c/mmole) and 7-[^{3}H]-*dl*-noradrenaline (4.0 c/mmole or 4.8 c/mmole) were obtained from New England Nuclear Corporation. [^{3}H]-Tyrosine injections consisted of 0.4 ml. of 0.1 N-HCl-0.75% NaCl solution and the [^{3}H]-noradrenaline injections were 0.4 ml. of 0.01 N-HCl-0.75% NaCl solution.

Student's *t* test was used in the evaluation of the data.

RESULTS

Effects of monoamine oxidase inhibition on the concentrations of noradrenaline in the hearts and catecholamines and 5-hydroxytryptamine in the brains of rats after repeated administration of CL-62375

After three injections of CL-62375 (50 mg/kg, intraperitoneally) at 3 hr intervals there was a decrease in the concentration of catecholamines in the heart (68%) and brain (17%) and a fall in the concentration of 5-hydroxytryptamine in the brain (66%) (Fig. 1). Pargyline (75 mg/kg, intraperitoneally) given 2 hr after the last injection of CL-62375 caused no significant rise in the concentration of noradrenaline in the hearts of the animals. Animals receiving only pargyline exhibited an increase of 45% in the noradrenaline concentration over that of the controls. In the brain there was no significant difference in catecholamine concentrations in the animals receiving CL-62375 plus pargyline in comparison with those given CL-62375 alone but there was only a small difference between the CL-62375-treated and the controls ; an increase was observed with pargyline alone. The brain 5-hydroxytryptamine in the animals receiving CL-62375 plus pargyline was somewhat higher than the controls. There was an increase of about four-fold in the 5-hydroxytryptamine concentration in the animals receiving only pargyline when compared with the controls.

Effects of monoamine oxidase inhibition on the concentration of noradrenaline in hearts of rats after repeated administrations of the 3-amino derivative of CL-62375 or a single administration of epsilon amino caproic acid

A structurally related compound, ethyl 3-amino-4-H-pyrrolo-[3,4-*c*]isoxazole-5(6H)-carboxylate was administered (75 mg/kg, intraperitoneally) to rats three times at 3 hr intervals. Other rats received a single injection of epsilon amino caproic acid, a catechol-

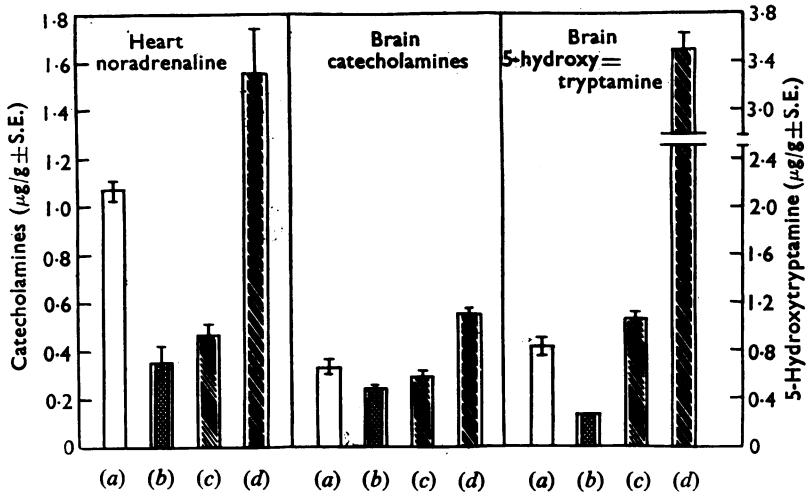

Fig. 1. Effects of monoamine oxidase inhibition on concentrations of noradrenaline in the heart and catecholamines and 5-hydroxytryptamine in the brain of rats after repeated administrations of CL-62375. CL-62375 (50 mg/kg, intraperitoneally) was administered three times at 3 hr intervals. Two hours after the last injection the monoamine oxidase inhibitor, pargyline (75 mg/kg, intraperitoneally), or its diluent was given. Tissues were removed 6 hr later for analysis. Each bar represents the average concentration (±S.E.) in the tissues of five to six treated animals and thirteen to sixteen control animals. (*a*), Controls ; (*b*), CL-62375 ; (*c*), CL-62375 + pargyline ; (*d*), pargyline.

amine-releasing drug (Lippmann & Wishnick, 1965), in a dose of 400 mg/kg, intraperitoneally, followed by two diluent injections at 3 and 6 hr. Three hours after the last treatment the monoamine oxidase inhibitor, pargyline (75 mg/kg, intraperitoneally), or its diluent was administered and the hearts were removed 3 hr later. As shown in Fig. 2, there was a 69% lowering in the concentration of noradrenaline in the animals treated with the 3-amino derivative of CL-62375 and a 67% reduction in those receiving epsilon amino caproic acid. The monoamine oxidase inhibitor reversed the lowering of the noradrenaline produced by epsilon amino caproic acid ; however, no rise in noradrenaline was observed after pargyline in the animals treated with the 3-amino derivative.

Effects of CL-62375 on uptake and release of administered noradrenaline in the rat heart

CL-62375 (50 mg/kg) was administered intraperitoneally to rats three times at 3 hr intervals ; 3 hr after the last dose, noradrenaline was given (2 mg/kg, intraperitoneally). Other animals received a single dose of α-methyl-meta-tyrosine (α-MMT), a drug which interferes with the ability of the tissue to store noradrenaline, at a dose of 400 mg/kg, intraperitoneally, 31 hr before the noradrenaline (Hess, Connamacker, Ozaki & Udenfriend, 1961 ; Spector, Sjoerdsma & Udenfriend, 1965). The animals were killed at the designated times and the noradrenaline in the hearts determined. In the animals receiving only the noradrenaline there was an increase in the noradrenaline concentration of almost two-fold after 1 hr (Fig. 3). The concentration was reduced at 4 hr and had returned to

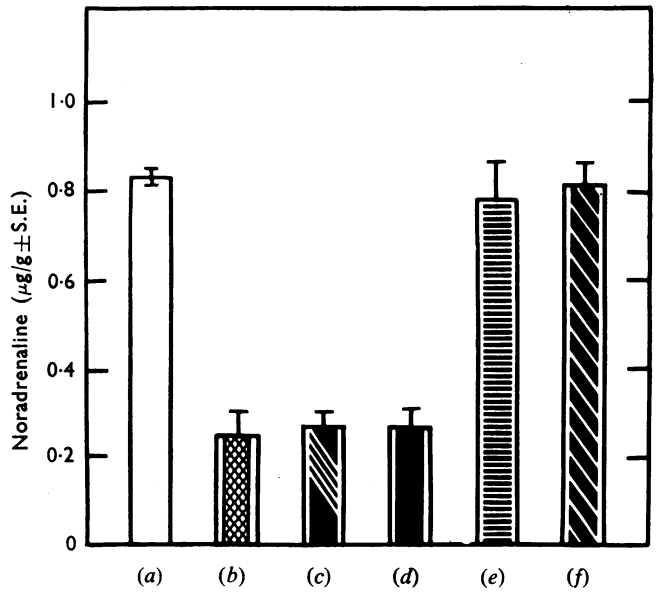

Fig. 2. Effects of monoamine oxidase inhibition on concentrations of noradrenaline in the hearts of rats after repeated administrations of the 3-amino derivative of CL-62375 or a single administration of epsilon amino caproic acid. Rats were injected with the 3-amino derivative of CL-62375 (75 mg/kg, intraperitoneally) three times at 3 hr intervals. Three hours after the last dose the monoamine oxidase inhibitor pargyline (75 mg/kg, intraperitoneally) or its diluent was administered. Tissues were removed 3 hr later for analysis. A single dose of epsilon amino caproic acid (400 mg/kg, intraperitoneally) was given 9 hr before the monoamine oxidase inhibitor. Tissues were removed 3 hr later for analysis. Each bar represents the average of the noradrenaline concentration (±S.E.) in the hearts of five to six treated animals and sixteen control animals. (*a*) Control ; (*b*), 3-amino derivative of CL-62375 ; (*c*), 3-amino derivative plus pargyline ; (*d*), epsilon amino caproic acid ; (*e*), epsilon amino caproic acid plus pargyline ; (*f*), pargyline.

normal at 6.5 hr. The animals receiving CL-62375 plus the noradrenaline showed a three fold increase in the noradrenaline concentration after 1 hr and the concentration had dropped at 6.5 hr to essentially that observed before the administration of noradrenaline. The concentration of noradrenaline in the animals treated with α-MMT plus noradrenaline rose five-fold after 1 hr and fell after 6.5 hr to a value about that observed in the animals treated with α-MMT before the administration of noradrenaline. Thus in both the CL-62375 and α-MMT-treated animals the concentrations of noradrenaline rose after the administration of noradrenaline but subsequently fell to the depleted levels.

Effects of a single administration of CL-62375 on uptake and release of [^{3}H]-tyrosine or [^{3}H]-noradrenaline in the rat heart

Rats received a single injection of CL-62375 (100 mg/kg, intraperitoneally), or its diluent as control, followed 30 min later by an intravenous injection of [^{3}H]-tyrosine or [^{3}H]-noradrenaline. The animals were killed 1.5 hr after the administration of CL-62375. Other rats were injected with [^{3}H]-noradrenaline 30 min before CL-62375 (100 mg/kg, intraperitoneally) or its diluent, was administered ; the animals were killed 1.5 hr after

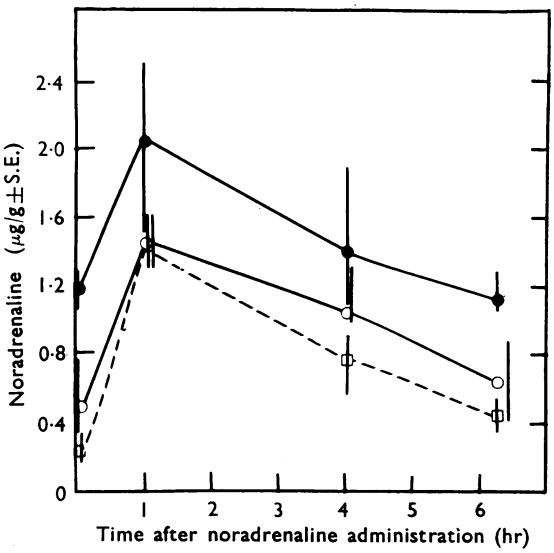

Fig. 3. Effects of CL-62375 on uptake and release of administered noradrenaline in the rat heart. CL-62375 (50 mg/kg, intraperitoneally) was given three times at 3 hr intervals (○——○). Three hours after the last treatment noradrenaline (2 mg/kg, intraperitoneally) was administered. A single dose of α-MMT (400 mg/kg, intraperitoneally) was administered 31 hr before the noradrenaline (□ - - - - □). Control, ●——●. There were five to six animals in each group. The ranges in values of noradrenaline concentrations are as indicated.

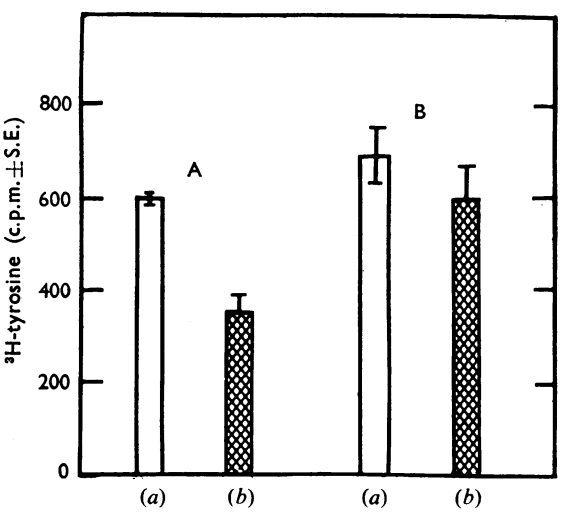

Fig. 4. Effects of a single administration of CL-62375 on uptake and release of [³H]-tyrosine in the rat heart. A: CL-62375 (100 mg/kg, intraperitoneally) was administered to rats (*b*) and controls received the diluent (*a*). Thirty minutes later, 70 μc. [³H]-tyrosine was injected into the tail vein. The animals were killed 1.5 hr after the administration of CL-62375 and the [³H]-tyrosine concentrations of the hearts were determined. Each bar represents the mean concentration (±S.E.) in four animals. B: [³H]-tyrosine (70 μc) was injected intravenously into rats followed 30 minutes later by an administration of CL-62375 (100 mg/kg, intraperitoneally) (*b*) or its diluent (*a*). The animals were killed 1.5 hr after the treatment with CL-62375 and the [³H]-tyrosine concentrations of the hearts were determined. Each bar represents the average concentration (±S.E.) in four animals.

the treatment with CL-62375. Treatment with CL-62375 before the [³H]-tyrosine caused a 42% reduction in the concentration of [³H]-tyrosine in the hearts (Fig. 4). There was no significant lowering in the concentration of [³H]-tyrosine in the hearts when CL-62375 was administered after [³H]-tyrosine. No significant reduction in the [³H]-noradrenaline concentration in the hearts was observed when CL-62375 was administered either before or after the [³H]-noradrenaline (Fig. 5).

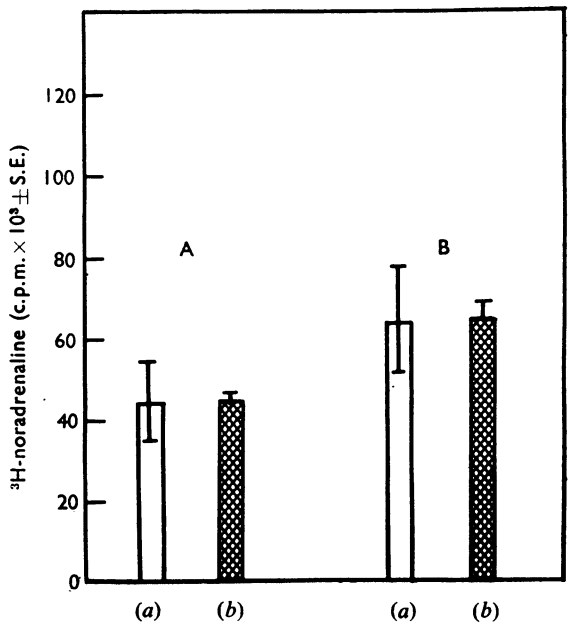

Fig. 5. Effects of a single administration of CL-62375 on uptake and release of [^{3}H]-noradrenaline in the rat heart. A: Rats were injected with CL-62375 (100 mg/kg, intraperitoneally) (*b*) and controls received the diluent (*a*). Thirty minutes later [^{3}H]-noradrenaline (40 μc) was administered intravenously. The animals were killed 1.5 hr after the treatment with CL-62375 and the [^{3}H]-noradrenaline concentrations of the hearts were determined. Each bar represents the average concentration (± S.E.) in four animals. B: [^{3}H]-Noradrenaline (40 μc) was given intravenously to rats followed 30 min later by an administration of CL-62375 (100 mg/kg, intraperitoneally) (*b*) or its diluent (*a*). The rats were killed 1.5 hr after the injection of CL-62375 and the [^{3}H]-noradrenaline concentrations of the hearts were determined. Each bar represents the average concentration (± S.E.) in four animals.

DISCUSSION

Ethyl 3-acetamido-4H-pyrrolo[3,4-*c*]isoxazole-5(6H) carboxylate [CL-62375] causes a lowering of the endogenous catecholamine and 5-hydroxytryptamine concentrations in tissues of the rat. Agents can decrease the endogenous biogenic amine concentrations by altering the storage mechanisms and by altering the synthesis of the amines. CL-62375 after a single administration (Lippmann & Wishnick, 1967), α-methyl-meta-tyrosine (α-MMT), which causes interference in the storage mechanisms and α-methyltyrosine (α-MT), which causes inhibition of catecholamine synthesis (Spector *et al.*, 1965; Weissman & Koe, 1965) are all similar in that they cause a large depletion of both heart and brain catecholamines and cause only a small or no decrease in the 5-hydroxytryptamine concentration in the brain. After repeated administration, CL-62375 (Lippmann & Wishnick, 1967) reduces catecholamine concentrations as does α-MT (Spector *et al.*, 1965); in contrast, CL-62375 causes a large decrease in the brain 5-hydroxytryptamine (Lippmann & Wishnick, 1967), whereas α-MT has no effect (Spector *et al.*, 1965). The depletion in the heart that is observed after repeated administration of CL-62375, or the structurally related compound ethyl 3-amino-4H-pyrrolo-[3,4-*c*] isoxazole-5(6H) carboxylate, is not reversed by administration of the monoamine oxidase inhibitor, pargyline (Figs. 1 and 2). In this respect, the compounds are similar to α-MT. In contrast, the depletions that are caused by both α-MMT (Hess *et al.*, 1961) and epsilon amino caproic acid (Fig. 2; Lippmann & Wishnick, 1965) are reversed by the monoamine oxidase treatment. The animals receiving CL-62375 differ in appearance from those receiving α-MT, because with α-MT sedation is observed (Spector *et al.*, 1965), where as no sedation is observed after treatment with CL-62375 (Lippmann & Wishnick, 1967).

The concentrations of noradrenaline in the hearts of animals receiving α-MMT or repeated injections of CL-62375 rise after administration of exogenous noradrenaline (Fig. 3). Thus, in these conditions neither of these drugs causes an alteration in the uptake of noradrenaline into the tissues. After 6.5 hr the concentrations of noradrenaline have returned to the depleted values, thereby indicating that the drugs are interfering with the binding of the noradrenaline. In this respect CL-62375 is similar to α-MMT, but differs from α-MT, because the latter has been shown (Spector *et al.*, 1965) to have no effect on the binding of noradrenaline. The finding that the monoamine oxidase inhibitor, pargyline, does not reverse the depleting effect might suggest an inhibition of synthesis. The results with the exogenously administered noradrenaline, however, indicate an alteration in the binding caused by CL-62375. Thus it seems that after repeated administration of CL-62375 there could be both an inhibition of synthesis and an alteration of the binding sites of noradrenaline. The depletion of serotonin caused by repeated administration of CL-62375 is reversed by pargyline, which suggests that the synthesis of 5-hydroxytryptamine is, in contrast to noradrenaline, not inhibited.

After a single administration, CL-62375 causes a hindrance of the uptake and availability of [^{3}H]-tyrosine, but not of [^{3}H]-noradrenaline, and does not cause an immediate release of tyrosine or noradrenaline (Figs. 4 and 5). The decreased concentrations of amines in the tissues observed after CL-62375 might be the result of a decreased availability of tyrosine in the tissues. The rate-limiting step in the synthesis of noradrenaline is the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (Levitt, Spector & Udenfriend, 1964). The availability of tyrosine, the substrate for this step, could therefore be a controlling factor in the formation of noradrenaline. A decreased uptake of the substrate for the rate-limiting step could possibly lead to a lowered concentration of noradrenaline. The initial effect of CL-62375 need not be reflected as a large fall in the noradrenaline concentration but rather as an interference in the synthesis of newly formed noradrenaline. Much of the noradrenaline found in the tissues, being present in a "tightly bound" form (Whitby *et al.*, 1961) might not be involved in the initial effects of CL-62375. Thus an early physiological effect, such as a decrease in blood pressure, might be produced in the absence of a large reduction in tissue noradrenaline concentration. Furthermore, only a relatively small amount of the noradrenaline present in tissues seems normally to be released after physiological stimulation of the adrenergic nerves (Crout, Muskus & Trendelenburg, 1962; von Euler, 1963; Kernell & Sedvall, 1964) and this might be the noradrenaline involved in the initial effect of CL-62375. Also, the bound noradrenaline is stored in a physiologically inert, chemically unchanged form (Potter & Axelrod, 1963). Activities which are mediated through the mobilization of "tightly bound" noradrenaline would not be expected to be altered in these conditions because of the presence of the unaffected stores. Also, a rise in blood pressure, observed after a comparatively large dose of exogenously administered noradrenaline, would not be affected for the rise in blood pressure would represent the additional amount of active noradrenaline made available to receptor sites in contrast to the normal amount available through synthesis.

That somewhat different physiological effects might be observed in animals receiving repeated administrations of CL-62375, is suggested by the observations obtained with the monoamine oxidase inhibitor and the exogenously administered noradrenaline. The

findings suggest that there is an alteration in the catecholamine binding sites, and thus responses mediated through the mobilization of the " tightly bound " noradrenaline, could be reduced because the levels of catecholamines would have been lowered. Because the major route of inactivation of noradrenaline is through binding and storage (Kopin. Hertting & Gordon, 1962), the inactivation of a large dose of exogenously administered noradrenaline would be decreased and a prolonged response could occur.

SUMMARY

1. After repeated administration of ethyl 3-acetamido-4H-pyrrolo [3,4-*c*] isoxazole-5(6H)carboxylate (CL-62375) the catecholamine concentrations in the heart and brain and the 5-hydroxytryptamine concentration in the brain of rats were decreased. Administration of a monoamine oxidase inhibitor, pargyline, to the treated animals did not cause a rise in the catecholamine concentrations in the heart and brain ; the concentration of 5-hydroxytryptamine in the brain showed an increase. The monoamine inhibitor alone caused an increase in each of the concentrations.

2. The structurally related 3-amino derivative of CL-62375 caused a decrease in the catecholamine concentration in the heart after repeated administration and the concentration was not raised by the subsequent treatment of the animals with a monoamine oxidase inhibitor.

3. In animals which had received repeated injections of CL-62375, there was a rise in the concentration of noradrenaline in the heart after administration of exogenous noradrenaline, but the concentration subsequently fell to below that of controls.

4. A single administration of CL-62375 caused a decrease in the uptake and availability of [^{3}H]-tyrosine, but not of [^{3}H]-noradrenaline, in the heart, and caused no immediate release of [^{3}H]-tyrosine or [^{3}H]-noradrenaline.

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