THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 178, No. 1

Printed in U.S.A.

# ULTRASTRUCTURAL CYTOCHEMISTRY AND PHARMACOLOGY OF 5-HYDROXYTRYPTAMINE IN ADRENERGIC NERVE ENDINGS. III. SELECTIVE INCREASE OF NOREPINEPHRINE IN THE RAT PINEAL GLAND CONSECUTIVE TO DEPLETION OF NEURONAL 5-HYDROXYTRYPTAMINE[1]

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Accepted for publication March 30, 1970

ABSTRACT

JAIM-ETCHEVERRY, GUILLERMO AND LUIS MARÍA ZIEHER: Ultrastructural cytochemistry and pharmacology of 5-hydroxytryptamine in adrenergic nerve endings. III. Selective increase of norepinephrine in the rat pineal gland consecutive to depletion of neuronal 5-hydroxytryptamine. J. Pharmacol. Exp. Ther. **178**: 42–48, 1971. Pineal glands of rats treated with *p*-chlorophenylalanine and desmethylimipramine were studied with biochemical and electron microscopic cytochemical techniques. Both drugs deplete the pool of pineal 5-hydroxytryptamine (5-HT) localized in the adrenergic fibers which innervate the gland. The cores demonstrating a cytochemical reaction for 5-HT disappeared from the glands of treated rats. However, catecholamines were detected cytochemically in the nerves. After treatment with either drug there was an increase in pineal norepinephrine (NE) as determined fluorimetrically. This increase, which was well established 24 hours after the first injection, was maintained by prolonging the treatment and was not affected by decentralization of both superior cervical ganglia. Under conditions in which pineal NE was increased, no variations were observed in the content of NE of other sympathetically innervated organs. This increase is most probably the result of an enhancement of NE synthesis triggered by disappearance of 5-HT from the vesicles. The results obtained support the hypothesis that both NE and 5-HT coexist in the nerve vesicles of pineal adrenergic fibers.

The pineal gland of the rat contains large amounts of 5-hydroxytryptamine (5-HT) which is localized both in its parenchymal cells, the pinealocyte, and in the nerve fibers supplying the gland (Pellegrino de Iraldi *et al.*, 1963). These fibers originate from neurons in the superior cervical ganglia and constitute the sole nervous supply to pineal cells and blood vessels. Since pineal norepinephrine (NE) is contained in these nerves, they are unique in that they store two biogenic amines (Owman, 1964). Several experimental approaches have confirmed that whereas NE is synthesized by the adrenergic neuron, 5-HT is formed in the pinealocyte and is subsequently taken up by adrenergic nerves, both compartments having different turnover rates (Neff *et al.*, 1969).

Recent pharmacologic, morphologic and cytochemical evidence show that intraneuronal pineal 5-HT is localized in vesicles (Bloom and Giarman, 1967, 1970; Jaim-Etcheverry and Zieher, 1968b; Pellegrino de Iraldi and Gueudet, 1969). Since adrenergic nerve vesicles store NE, we have proposed that both 5-HT and NE coexist within these vesicles (Jaim-Etcheverry and Zieher, 1968b). This "common vesicular storage" mechanism has also been found to be present in the adrenergic vesicles of the vas deferens under special experimental conditions (Zieher and Jaim-Etcheverry, 1971).

The experiments reported here were initiated

Received for publication September 24, 1970.
[1] This work was supported by Research Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas—Fondo de Farmacología, Ley 17.189, art. 9-República Argentina and National Institutes of Health, Grant 5-RO 1-NS-06953-05 NEUA.

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to provide more direct evidence of the existence of such a storage mechanism by studying the changes in pineal NE which follow the removal of 5-HT from the vesicles. This was achieved either by inhibiting 5-HT synthesis with DL-*p*-chlorophenylalanine (Koe and Weissman, 1966) or by blocking its uptake into the nerves with desmethylimipramine (Neff *et al.*, 1969).

METHODS. Male Wistar rats weighing 200 to 250 g were placed, at least three weeks before being used, in a controlled environment in which fluorescent light was kept on from 7.00 A.M. to 7.00 P.M. (a cycle of 12 hours of light and 12 hours of darkness). DL-*p*-Chlorophenylalanine (PCP; Pfizer Inc., New York, N.Y.) or its methyl ester (H69/17, AB. Hässle, Göteborg, Sweden) were dissolved, respectively, according to Koe and Weissman (1966) or in saline. Similar results have been obtained with both compounds. In acute experiments animals were killed two hours after receiving a single i.v. injection of PCP, 100 mg/kg. In other experiments rats were given one, two or three injections (350 mg/kg i.p.) at 24-hour intervals and were killed 24 hours after the last injection.

Groups of rats receiving desmethylimipramine (DMI), 20 mg/kg i.p., dissolved in saline, were killed 2 or 24 hours later. Other animals were given DMI, 20 mg/kg, on the first day followed by 10 mg/kg at 24 hours. Some of these were killed 24 hours later (48 hours after the first injection), whereas the remainder received another injection of 10 mg/kg and were killed 72 hours after the first injection.

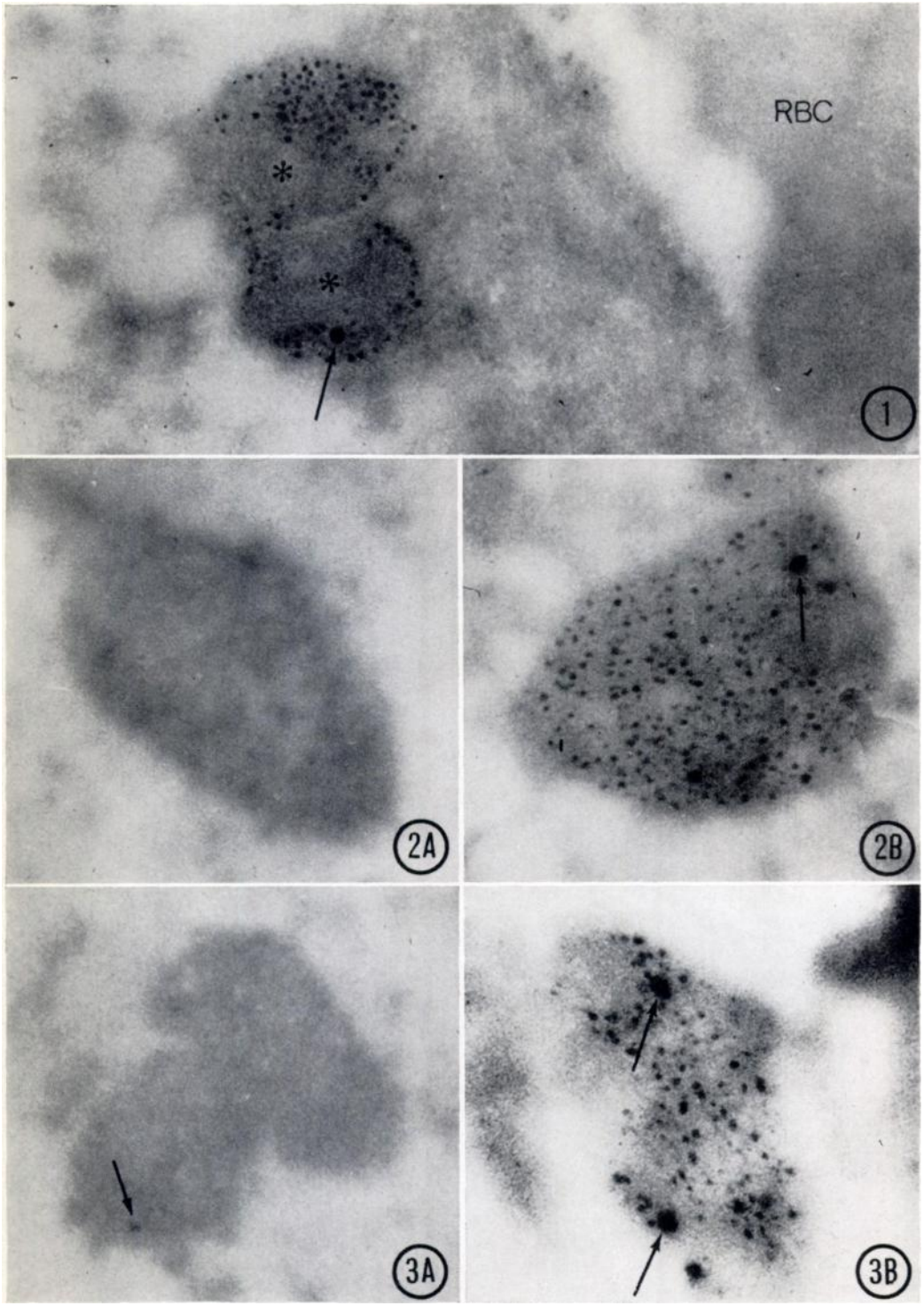
In a group of rats, both superior cervical ganglia were decentralized by transecting the preganglionic trunk under light ether anesthesia. Forty-eight hours after the operation, the rats received PCP methyl ester (350 mg/kg i.p.) and were killed 24 hours later. Sham-operated rats were used as controls in this experiment.

Special care was taken to make the injections approximately at the same moment of the lighting cycle and to decapitate the animals between 2 and 3 P.M. to avoid variations in amine content of the tissues studied. Groups of rats injected at the same time intervals with the appropriate solvents were used as controls.

*Electron microscopy.* Cytochemical methods for the ultrastructural demonstration of biogenic amines were performed as described previously (Zieher and Jaim-Etcheverry, 1971; Jaim-Etcheverry and Zieher, 1968a). In some experiments the fixative and washing solutions were prepared in 0.2 M cacodylate buffer, pH 7.2, whereas in others 0.1 M phosphate buffer, pH 7.3, was used. The procedure involved treatment of one-half of each gland with glutaraldehyde and potassium dichromate (GD reaction) which depicts cellular sites containing both catecholamines and 5-HT. The other half of each gland was placed in formaldehyde prior to glutaraldehyde-dichromate treatment (FGD reaction), resulting in a disappearance of catecholamine reactive sites but no change in 5-HT sites. In analyzing the results of these experiments, special attention has been paid to the fact that both halves of a same gland, each processed with a different technique, were being studied. From control, PCP- and DMI-treated groups, 10 glands were processed in the same way. Electron micrographs corresponding to perivascular processes, which are easily identifiable, were analyzed by unaware observers who recorded the presence or absence of granular reactive material which is the only structure present in the sections.

*Amine assays.* Norepinephrine was determined by the method of Häggendal (1963) and 5-HT by a modification of the method of Andén and Magnusson (1967), as described previously (Zieher and Jaim-Etcheverry, 1971). Mean recoveries were 85 and 90% for NE and 5-HT, respectively. The significance of results was determined by Student's *t* test.

RESULTS. ELECTRON MICROSCOPY. Figure 1 shows the presence of 5-HT within nerve vesicles in a pineal ending according to the FGD technique in a normal gland. Both small (300–500 Å diameter) and large (700–1000 Å) granulated vesicles store the amine (Jaim-Etcheverry and Zieher, 1968b). The administration of PCP resulted in the disappearance of the cores revealed by the FGD procedure indicating depletion of vesicular 5-HT from pineal nerves (fig. 2A). The fact that the cores reacting with the GD technique in the other half of the gland were unaffected confirms that a catecholamine, probably NE, is not removed from its storage sites in the nerves by PCP (fig. 2B). After administration of DMI, the FGD technique failed to reveal reactive cores in the majority of pineal perivascular processes, although approximately 10% of such processes can be positively identified as nerve terminals due to the persistence of a few 5-HT reactive sites (one to five per profile) (fig. 3A). The other half of the gland processed with the GD technique has a normal cytochemical appearance (fig. 3B). These observations indicate that both PCP and DMI produce a depletion of neuronal 5-HT without diminution in the reaction given by vesicular catecholamines.




FIGS. 1–3.

TABLE 1

*5-Hydroxytryptamine and norepinephrine content of the pineal gland of the rat after PCP and DMI treatments*[a]

Results are expressed in micrograms per gram of wet weight and are mean values ± S.E. for four to five experiments. Percent changes are also given.

| | Content after: | | | |
|---|---|---|---|---|
| | PCP | %Δ | DMI | %Δ |
| | μg/g | | μg/g | |
| 5-Hydroxytryptamine | | | | |
| Controls | 55.23 ± 2.96 | | | |
| 2 hr | 17.37 ± 2.60[b] | −69 | | |
| 72 hr | 8.74 ± 0.73[b] | −84 | 52.62 ± 3.18 | −5 |
| Norepinephrine | | | | |
| Controls | 6.96 ± 0.24 | | | |
| 2 hr | 8.22 ± 0.68 | +18 | 7.56 ± 0.58 | +8 |
| 24 hr | 17.94 ± 1.86[b] | +158 | 15.04 ± 0.90[b] | +117 |
| 48 hr | 11.87 ± 0.49[b] | +71 | 15.62 ± 1.24[b] | +125 |
| 72 hr | 11.29 ± 0.82[b] | +62 | 13.34 ± 1.53[b] | +92 |

[a] Data corresponding to dosage and time schedules for administration of drugs is given in METHODS.

[b] $P < .001$.

DRUG-INDUCED CHANGES IN AMINE CONTENT. *1) Pineal gland.* Changes in the 5-HT and NE content of the pineal gland produced by PCP and DMI are shown in table 1. Pineal 5-HT was diminished by approximately 70% two hours after injection of PCP (100 mg/kg i.v.). This depletion was even more pronounced when the amine was assayed after a 72-hour treatment with PCP. The administration of DMI for 72 hours produced no significant decrease in total pineal 5-HT. With respect to pineal NE, a slight but nonsignificant increase was observed two hours after administration of either drug. However, 24 hours after injection of either PCP or DMI, there was a striking increase in pineal NE which was maintained by prolonging the treatment for 48 or 72 hours. PCP produced a maximal increase in pineal NE content of 158% above control levels at 24 hours; when the treatment was maintained for 48 or 72 hours, NE levels remained around 70% above controls. DMI caused a similar increase in pineal NE at 24, 48 and 72 hours (approximately 100% above controls).

The effects of bilateral decentralization of the superior cervical ganglia on the content of 5-HT and NE in the pineal gland are shown in table 2. The concentration of both amines was not

TABLE 2

*Effects of decentralization of the superior cervical ganglia on pineal norepinephrine and 5-hydroxytryptamine levels after treatment with PCP*[a]

Results are expressed in micrograms per gram of wet weight and are mean values ± S.E. of six experiments. Percent changes are also given.

| | Norepinephrine | %Δ | 5-Hydroxytryptamine | %Δ |
|---|---|---|---|---|
| | μg/g | | μg/g | |
| Controls | 5.88 ± 0.74 | | 40.70 ± 4.35 | |
| Decentralized, 72 hr | 5.65 ± 0.33 | | 46.45 ± 7.61 | |
| PCP, 24 hr | 11.81 ± 2.11[b] | +100 | 7.30 ± 0.59[b] | −82 |
| Decentralized, 48 hr + PCP, 24 hr | 10.33 ± 0.95[c] | +82 | 8.14 ± 1.95[c] | −80 |

[a] Both superior cervical ganglia were decentralized by transection of the preganglionic trunk and 48 hours later, rats were injected with saline or PCP, 350 mg/kg i.p., and killed after 24 hours.

[b] $P < .001$ when compared with control values.

[c] $P < .001$ when compared with values of decentralized animals.

FIG. 1. Electron micrograph of sympathetic fibers in the pineal gland of the normal rat processed with the cytochemical technique for the demonstration of 5-HT (FGD reaction). Both nerve fibers are localized in the perivascular space of the gland in the vicinity of a blood vessel (RBC, red blood cell). Two types of reactive granules may be distinguished by their size: small granules of 200 to 300 Å in diameter and larger dense precipitates of 500 to 600 Å (↑). These densities can be matched with the dense cores of the small and large granulated vesicles observed with conventional techniques in these endings. Asterisks indicate the sites probably occupied by mitochondria in the endings. ×39,000.

FIG. 2. Sympathetic fibers in the perivascular space of the pineal gland from a rat treated with *p*-chlorophenylalanine (three 350 mg/kg i.p. doses, 72 hours). In the process in A, no reactive cores are observed when half of the gland is treated with the cytochemical technique for 5-HT (FGD reaction). ×40,000. In B, both small and large (↑) reactive sites observed in the other half of the gland processed with the GD reaction indicate the presence of catecholamines. ×48,000.

FIG. 3. Electron micrographs of sympathetic endings in the perivascular space of the pineal gland of a rat treated with desmethylimipramine (20 mg/kg and two 10 mg/kg i.p. doses, 72 hours). In A, no reactive sites are observed in the majority of perivascular processes when half of the gland is treated with the FGD technique for 5-HT. Rarely, few small reactive sites as the one shown in the picture (↓) remain in the ending and allow its positive identification. ×60,000. In the ending shown in B, small and large (↑) reactive sites appear with the GD procedure and reveal the presence of catecholamines. ×52,000.

TABLE 3

*Norepinephrine content of several sympathetically innervated organs after administration of PCP and DMI*[a]

Results are expressed in micrograms per gram of wet weight and are mean values ± S.E. of four to five experiments.

| | Heart | Salivary Glands | Vas Deferens | Spleen |
|---|---|---|---|---|
| Controls | 0.985 ± 0.04 | 1.60 ± 0.09 | 14.23 ± 1.25 | 0.469 ± 0.04 |
| PCP, 2 hr | 1.008 ± 0.14 | 1.74 ± 0.18 | 15.03 ± 1.64 | 0.415 ± 0.03 |
| PCP, 72 hr | 0.915 ± 0.13 | 1.56 ± 0.24 | 14.02 ± 2.14 | 0.470 ± 0.03 |
| DMI, 72 hr | 1.027 ± 0.04 | 1.68 ± 0.07 | | |

[a] Data corresponding to dosage and time schedules for administration of drugs is given in METHODS.

affected by preganglionic denervation, a finding which is in line with results showing the lack of effect of decentralization on peripheral NE levels (Fischer and Snyder, 1965). The increase in NE as well as the depletion of 5-HT produced by PCP injection (350 mg/kg i.p., 24 hours) was of similar magnitude in both normal and decentralized pineal glands.

*2) Other organs.* The concentration of NE in peripheral stores other than the pineal did not change in animals treated as described above with PCP or DMI (table 3).

DISCUSSION. The administration of either PCP or DMI to rats results in the depletion of neuronal 5-HT from the pineal gland; this is reflected in the disappearance from the nerves of the cores reacting with the FGD procedure. The persistence of dense deposits with the GD method indicates that NE is not reduced. The mechanisms by which these drugs deplete 5-HT are entirely different. Whereas PCP blocks tryptophan hydroxylase (Koe and Weissman, 1966) and decreases pineal 5-HT rapidly and profoundly, DMI blocks its transport through the axonal membrane (Eccleston *et al.*, 1968). In the case of DMI, 5-HT disappears only from the nerves, as was demonstrated by the kinetic and fluorescence histochemical studies of Neff *et al.* (1969) and is now confirmed cytochemically at the ultrastructural level. This selective neuronal depletion of 5-HT indicates that the neural store of 5-HT is maintained by this uptake process. The fact that 5-HT disappears from the nerves without a decrease in pineal 5-HT content reveals the capacity of the pinealocyte to store the amine which is normally taken up by the nerves.

The ability to incorporate 5-HT shown by pineal nerves is common to other sympathetic postganglionic fibers (Owman, 1964; see Zieher and Jaim-Etcheverry, 1971). However, although exogenous 5-HT is taken up even when given in tracer doses (Eccleston *et al.*, 1968), very high concentrations of the amine must be offered to the endings to allow its cytochemical detection within adrenergic nerve vesicles without an associated depletion of endogenous NE (*e.g.*, approximately $5.6 \times 10^{-4}$ M in the case of vas deferens slices incubated *in vitro* with exogenous 5-HT) (Zieher and Jaim-Etcheverry, 1971). Therefore, the concentration of 5-HT normally present around pineal endings is probably of this order of magnitude, which is highly possible considering the large amount of 5-HT metabolized in the gland.

On the basis of cytochemical studies in rat pineal nerves showing that almost all vesicles had a core giving the reaction for 5-HT, the possibility arose that both 5-HT and NE coexist in the same vesicle (Jaim-Etcheverry and Zieher, 1968b), an assumption which received some experimental support from *in vitro* studies in the rat vas deferens (Zieher and Jaim-Etcheverry, 1971). If pineal nerve vesicles are similar to those of other adrenergic endings, the only difference being that 5-HT has accumulated in them because of the special conditions of their milieu, it is conceivable that the partial emptying of such vesicles by depletion of their 5-HT will produce a compensatory increase in NE due to the availability of storage sites (See Weiner, 1970). This hypothesis was confirmed in this study by the marked and selective increase in pineal NE which occurs after the depletion of 5-HT from pineal nerves; it is further supported by the increase in neuronal 5-HT which occurs after NE depletion (Zweig and Axelrod, 1969). However, both conditions are not exactly similar since the increase in neuronal 5-HT probably depends on the avail-

ability of the amine for uptake, which might vary after depletion of NE. The increase in pineal NE observed is in contrast with previous observations that PCP treatment, although depleting pineal 5-HT, does not modify the content of NE of the gland (Pellegrino de Iraldi and Gueudet, 1969; Bloom and Giarman, 1970) and that the cores observed with the GD reaction in pineal nerves of PCP-treated rats are less dense than those in control endings (Pellegrino de Iraldi and Gueudet, 1969). These results emphazise the danger of drawing conclusions on the mechanism of amine storage simply from the observation of changes in density of the cytochemical product, which is obviously influenced by many factors involved in the handling of tissues for electron microscopy.

The rise in pineal NE might result either from decreased metabolic degradation or release, increased reuptake or enhanced synthesis. The lack of effect of both drugs on NE levels in other organs as well as the increase in pineal NE even when its reuptake is blocked with DMI make improbable the first three possibilities and rule out a probable direct effect on NE concentration. Therefore, the increase must be due to an enhancement of NE synthesis which most likely is regulated at the rate-limiting step, tyrosine hydroxylation (Levitt *et al.*, 1965). Two different mechanisms might account for an increase in tyrosine hydroxylase activity. First, the synthesis of new enzymatic protein, a mechanism which reflects a long adaptation phenomenon and requires an intact preganglionic innervation (Mueller *et al.*, 1969a,b). This is unlikely to be the mechanism responsible for the increase in pineal NE after the depletion of neuronal 5-HT, since this increase is not abolished by bilateral decentralization of the superior cervical ganglia or by the inhibition of protein synthesis by actinomycin D or cycloheximide (unpublished results). The second mechanism involves the release of tyrosine hydroxylase from the feedback inhibition exerted by cytoplasmic catecholamines. When vesicular stores are partially emptied, the incorporation of cytoplasmic catecholamines into the vesicles reduces their concentration and tyrosine hydroxylation is enhanced (see Weiner, 1970). After disappearance of neuronal 5-HT, cytoplasmic catecholamines are most probably incorporated in the partially depleted vesicles and tyrosine hydroxylase is liberated from its control. This basic mechanism of incorporation of one amine in the vesicles after depletion of the other is supported by the results of Zweig and Axelrod (1969) who studied the increase in 5-HT after depletion of pineal NE. Apart from this known regulating mechanism, the possibility that tyrosine hydroxylase is also controlled by the concentration of cytoplasmic 5-HT in pineal nerves, should be considered. The depletion of this pool would thus lead to an enhanced tyrosine hydroxylation and the increase in pineal NE would result from the availability of storage sites created by the disappearance of vesicular 5-HT. The time lag observed between the disappearance of neuronal 5-HT and the rise in NE (*i.e.*, two hours after PCP, 5-HT is depleted by 70% whereas NE is not significantly increased) is comparable to that found in the reverse situation (depletion of NE followed by increase in 5-HT) studied by Zweig and Axelrod (1969).

The "common vesicular storage" mechanism provides a structural basis for the participation of postganglionic sympathetic nerves in the control of pineal indole metabolism. Recent evidence indicates that this function is mediated by NE which acts as the neural transmitter in the gland, 5-HT having opposite actions on pineal cell receptors (Wurtman *et al.*, 1969). However, NE and 5-HT which are stored within nerve vesicles are most probably liberated together during nerve activity, the physiologic effect of sympathetic nerve stimulation thus being the result of the relative amounts of NE and 5-HT simultaneously released. This ratio in turn reflects the vesicular 5-HT/NE ratio, which may be modified by changes in the concentration of parenchymal 5-HT or by the 24-hour cyclic variations in pineal NE and 5-HT levels.

CONCLUSIONS. 5-HT contained within nerve vesicles of adrenergic fibers innervating the pineal gland of the rat is depleted by treatment of the animals with *p*-chlorophenylalanine or desmethylimipramine. This depletion causes an increase in pineal NE which is not observed in other sympathetically innervated organs. This increase in pineal NE which is not affected by previous decentralization of the neurons supplying the gland, adds further support to the hypothesis that both amines coexist in amine storage vesicles in rat pineal nerves.

ACKNOWLEDGMENTS. We are grateful to Professor E. De Robertis and to Dr. M. Vogt for

critical reading of the manuscript, to Dr. S. Z. Langer for his interest and helpful suggestions in the course of this study and to Mrs. Cristina Llorente for her excellent technical assistance. The supply of *p*-chlorophenylalanine kindly given by Dr. A. Weissman, Pfizer Inc. and of desmethylimipramine (Pertrofran) by Geigy Argentina, Buenos Aires, is gratefully acknowledged.

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