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ULTRASTRUCTURAL CYTOCHEMISTRY AND PHARMACOLOGY OF 5-HYDROXYTRYPTAMINE IN ADRENERGIC NERVE ENDINGS. II. ACCUMULATION OF 5-HYDROXYTRYPTAMINE IN NERVE VESICLES CONTAINING NOREPINEPHRINE IN RAT VAS DEFERENS[1]

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ABSTRACT

ZIEHER, LUIS MARÍA AND GUILLERMO JAIM-ETCHEVERRY: Ultrastructural cytochemistry and pharmacology of 5-hydroxytryptamine in adrenergic nerve endings. II. Accumulation of 5-hydroxytryptamine in nerve vesicles containing norepinephrine in rat vas deferens. J. Pharmacol. Exp. Ther. **178**: 30–41, 1971. To study the localization of 5-hydroxytryptamine (5-HT) in the adrenergic nerve endings of rat vas deferens, biochemical and electron microscopic cytochemical techniques were applied to slices from the organ incubated *in vitro* with different concentrations of the amine. Exogenous 5-HT was detectable cytochemically within nerve fibers when its concentration in the medium was 5.6×10^{-4} M or higher. Cell fractionation studies indicated that the amine was concentrated in the microsomal fraction paralleling the distribution of endogenous norepinephrine. Moreover, norepinephrine was depleted from intact slices and from the microsomal fraction depending on the 5-HT accumulated, which in turn was a function of the exogenous 5-HT in the medium. The participation of adrenergic nerves in the process of 5-HT accumulation was confirmed by the reduction in this incorporation after chemical sympathectomy with 6-hydroxydopamine. These results indicate that under special conditions, exogenous 5-HT is incorporated into adrenergic nerves where it may be detected cytochemically without a significant depletion of endogenous norepinephrine. The existence of a "common vesicular storage" mechanism by which both amines are stored in the same morphologic entity in adrenergic postganglionic fibers is supported by these studies.

The capacity of peripheral adrenergic nerve fibers to store 5-hydroxytryptamine (5-HT) is demonstrated by the presence of the amine in the adrenergic nerve endings found in the pineal gland of the normal rat (Bertler *et al.*, 1964; Pellegrino de Iraldi *et al.*, 1963, 1965). Pineal 5-HT originates in the pinealocytes, which are characterized by a very active indole synthesis and seems to be subsequently taken up by the nerves (Owman, 1964; Neff *et al.*, 1969). Since norepinephrine (NE) is also present in pineal nerves (Pellegrino de Iraldi and Zieher, 1966; Wurtman and Axelrod, 1966) they constitute the only known example of sympathetic endings storing two biogenic amines. However, the incorporation and retention of 5-HT is not a unique feature of pineal endings since other adrenergic nerves show a similar ability when they are exposed either *in vivo* or *in vitro* to environments in which the concentration of 5-HT has been experimentally increased (Owman, 1964; Taxi and Droz, 1966; Eccleston *et al.*, 1968; Jaim-Etcheverry and Zieher, 1969a; Snipes *et al.*, 1968; Thoa *et al.*, 1969; Taxi, 1969).

Several studies have been devoted to determining the localization of neurotransmitters at the ultrastructural level, and strong evidence has accumulated indicating that the granular vesicles, characteristic of postganglionic sympathetic axons, constitute one of the storage sites of NE (Pellegrino de Iraldi and De Robertis, 1961;

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see Tranzer *et al.*, 1969; Jaim-Etcheverry and Zieher, 1969c; Bloom, 1970). On the basis of combined morphologic and pharmacologic evidence it has been proposed that 5-HT also resides in the cores of granular vesicles in pineal nerves (Bloom and Giarman, 1967; Bloom, 1968). This assumption was confirmed by the direct demonstration of the amine in the cores with a specific cytochemical reaction at the electron microscope level (Jaim-Etcheverry and Zieher, 1968b). This finding has since received support from combined cytochemical and pharmacologic experiments (Jaim-Etcheverry and Zieher, 1969c; Pellegrino de Iraldi and Gueudet, 1969; Bloom and Giarman, 1970). Using this reaction we have recently studied the intraneuronal fate of 5-HT administered *in vivo* in the nerves of the vas deferens. Even after very high doses of 5-HT no reaction product was detected within the vesicles. However, 5-HT was incorporated into them when the same dose was injected after depleting the endogenous NE (Jaim-Etcheverry and Zieher, 1969a).

In an attempt to explain the presence of cytochemically detectable 5-HT within adrenergic nerve vesicles in pineal nerves of the normal rat, experiments *in vitro* were designed to achieve higher concentrations of 5-HT around the terminals of the vas deferens than those probably attained by administration of the amine *in vivo*. The cytochemical pattern as well as the biochemical changes and subcellular distribution of NE and 5-HT in slices of vas deferens incubated in the presence of various concentrations of exogenous 5-HT have been analyzed. The participation of adrenergic nerves in this process has been studied by pretreating the rats with 6-hydroxydopamine, a drug which produces destruction of the nerves and results in a chemical sympathectomy of the organ (Thoenen and Tranzer, 1968). Moreover, evidence has been obtained supporting the existence of a storage mechanism for NE and 5-HT which we have proposed in preliminary studies, *i.e.*, their coexistence in the amine storage vesicles.

METHODS. Adult Wistar rats of 200 to 300 g b.wt. were used. They were decapitated between 1 and 2 P.M. to eliminate possible diurnal variations in amine content. The vasa deferentia were quickly excised and placed in cold McEwen's (1956) salt solution (NaCl, 7.6 g; KCl, 0.42 g; $CaCl_2$, 0.31 g; $NaH_2PO_4 \cdot 2H_2O$, 0.18 g; $NaHCO_3$, 2.1 g; glucose, 2.0 g; sucrose, 4.5 g and distilled water to make 1 liter). The peritoneal coat of the vas deferens with its blood vessels was carefully removed and transverse slices of about 0.5 to 1.5 mm thick were cut with a razor blade. The 5 mm corresponding to the urethral end of the organ were discarded and both the urethral and middle thirds were used for incubation.

Some experiments were carried out with the vasa deferentia of rats treated with 6-hydroxydopamine (6-OHDA). The drug (2,4,5-trihydroxyphenylethylamine hydrobromide, F. Hoffmann-La Roche & Co., Basel, Switzerland) was dissolved just before use in 0.001 N HCl gassed with nitrogen and at 4°C to retard oxidation. Two i.v. injections of 68 mg/kg of 6-OHDA base were given at a 48-hour interval and the animals killed 48 hours after the last injection. Slices of vas deferens were incubated as described below.

Incubation procedure. The slices obtained from vasa deferentia of 8 to 10 rats were pooled and distributed between the incubation flasks (100–150 mg of tissue in each) which contained 20 ml of McEwen's solution (final volume maintained throughout the incubation). The incubation was performed in a metabolic shaker at 37°C under a 95% O_2 and 5% CO_2 atmosphere. After 15 minutes incubation to equilibrate the tissue to the conditions *in vitro*, 5-hydroxytryptamine creatinine sulfate (Sigma Chemical Company, St. Louis, Mo.) was added to the incubation medium to achieve various final concentrations which are expressed as the free base. The tissue was incubated in two changes (20 minutes each) of medium plus amine. At the end of the incubation period the slices were rinsed several times over a 5-minute period with amine-free medium and incubated for a further 15 minutes in McEwen's solution without additions. This period has been found sufficient to wash out the amine incorporated *in vitro* into tissues and which is present in the extracellular space. This allows a more accurate study of the neuronal incorporation although during this period there is also a slight spontaneous release of intraneuronal amines (Jonsson *et al.*, 1969). The slices were blotted carefully with filter paper and processed for electron microscopy, cell fractionation or the determination of amine content.

Electron microscopy. Slices incubated in different concentrations of 5-HT were placed in the aldehyde fixatives for an initial period of 15 minutes. Blocks of approximately 1 mm^3 were trimmed from the outer muscular layer and returned to the fixative solutions for the total times given below. Two different processing schedules were used:

a) 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for four to six hours, washing in 0.3 M sucrose in the same buffer and treatment with a 2.5% solution of potassium dichromate and 1%

sodium sulfate in 0.2 M acetate buffer, pH 4 to 4.2, for four to six hours (GD reaction). b) 8% formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 24 hours, washing in 0.3 M sucrose and subsequent treatment in glutaraldehyde and dichromate solutions as in a) (FGD reaction).

After dehydration in ethanol, the blocks were transferred to propylene oxide and embedded in Epon 812. Thin sections cut with glass knives were mounted on uncoated copper grids and observed unstained in a Siemens Elmiskop I with double condenser operating at 80 kV.

The cytochemical procedure used, based on the light microscopic findings of Wood (1967), has been recently introduced to differentiate between catecholamines and 5-HT at the fine structural level (Jaim-Etcheverry and Zieher, 1968a). It has been applied to several amine-containing systems and analyzed *in extenso*. In general, the procedure involves treatment with glutaraldehyde and potassium dichromate (GD reaction) and depicts cellular sites containing both catecholamines and 5-HT. The fixation in formaldehyde prior to glutaraldehyde-dichromate treatment (FGD reaction) results in the disappearance of the reaction given by catecholamines, whereas it leaves unaffected that of structures containing 5-HT. The results have been analyzed as already described in detail in the first paper of this series (Jaim-Etcheverry and Zieher, 1969a). Slices from five different experiments were processed for cytochemical studies. In all the experiments and for each of the various concentrations of 5-HT used, one slice was processed with the GD and another one with the FGD reaction; therefore, 10 blocks (5 processed with the GD and 5 with the FGD technique) were obtained for each of the concentrations of 5-HT tested. From each of these blocks, three to five grids were studied under the electron microscope. Processes between smooth muscle cells were observed and photographed at final magnifications of ×15,000 to 20,000. The presence or absence of granular reactive material was recorded since with both cytochemical procedures used, only the clusters of dense granules which result from the amine-reagents interaction are apparent. The structures usually observed in the endings (membranes, mitochondria, etc.) are not visible with these techniques. Nerve endings are thus unidentifiable when the granules do not react. This is the reason why only "all or none" results can be recorded and it is impossible to grade the results quantitatively.

Cell fractionation procedures. Slices obtained after incubation were weighed and placed in cold 0.32 M sucrose containing the monoamine oxidase inhibitor tranylcypromine sulfate 10^{-4} M (Smith Kline and French Laboratories, Philadelphia, Pa.). Approximately 80 to 120 mg of tissue were homogenized in 3 ml of sucrose in a glass homogenizer with a tight fitting Teflon pestle in an ice bath. All fractionations were carried out at 4°C. The homogenization was performed in two periods of one minute each (11 strokes) with one-minute interval. This scheme was selected in preliminary experiments since the recovery of NE in particulate fractions could not be changed by increasing homogenization time. The volume was made up to 5 ml and the homogenate centrifuged at 900 × *g* for 10 minutes in a Sorvall RC2 B refrigerated centrifuge. The pellet was rehomogenized for 30 seconds in 3 ml of sucrose and again centrifuged at 900 × *g* for 10 minutes to obtain a sediment (coarse fraction). The supernatants from both centrifugations were pooled and centrifuged for 20 minutes at 11,500 × *g*. The supernatant from this centrifugation was separated from the pellet (mitochondrial fraction) and centrifuged at 100,000 × *g* for 30 minutes in the 40 rotor of a Spinco ultracentrifuge to obtain the microsomal fraction and the high-speed supernatant. The total homogenate as well as the different particulate fractions and the high-speed supernatant were extracted for NE, 5-HT and protein assay.

Biochemical assays. NE was extracted in 0.4 N perchloric acid and purified by column chromatography on Dowex 50W-X4 resin. The amine was eluted from the column with hydrochloric acid and measured according to Häggendal (1963). The fluorescence of samples was read at 400 and 515 mμ, corresponding to the excitation and emission wavelengths, respectively. 5-HT was extracted in perchloric acid containing ascorbic acid and ethylenediamine tetraacetic acid and the extract was purified in a column of Amberlite IRP 64 (2.3 × 20 mm). The eluate (0.9 ml of 1 N HCl) was processed according to Andén and Magnusson (1967) and the fluorescence read in an Aminco-Bowman spectrofluorophotometer at excitation and emission wavelengths of 300 and 545 mμ, respectively. Mean recoveries were 86 and 91% for NE and 5-HT, respectively. Protein was assayed in the perchloric acid precipitate of amine extracts by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Monoamine oxidase (MAO) activity was determined according to McCaman *et al.* (1965). Preliminary experiments were carried out to compare the activity of the enzyme in vas deferens homogenates and in small slices of the organ. The deaminating activity of the slices, although only 32% of that in the homogenate, was sufficiently high to study its experimental modifications. Therefore, vas deferens slices were used to analyze the effect of 5-HT on MAO activity in an attempt to reproduce as closely as possible the system used in the rest of the experiments. A slice of vas deferens

weighing 1 to 2 mg was placed in a microtube with 25 μl of buffer substrate solution. This solution contained 0.8 mM 2-^{14}C-5-hydroxytryptamine hydrogen oxalate (specific activity 3.81 mc/mmol, New England Nuclear Corporation, Boston, Mass.) in 0.1 M potassium phosphate buffer, pH 7.2. The tubes were incubated at 37°C for 30 minutes, and the reaction was stopped by the addition of 3 μl of 3 N HCl. The products formed by deamination were extracted in 100 μl of ethyl acetate and 50 μl of the upper phase were transferred to a counting vial and mixed with 1 ml of absolute methanol and 10 ml of a scintillator toluene solution (4 g of 2,5-diphenyloxazole per liter of toluene). For studying the effects of different concentrations of 5-HT on MAO activity and in order to reproduce the conditions obtained with incubation of slices, unlabeled amine was added to the buffer substrate solution. The concentrations tested were 5.6×10^{-5}, 5.6×10^{-4} and 5.6×10^{-3} M which correspond to 10, 100 and 1000 μg/ml of 5-HT base. These were the final concentrations of unlabeled 5-HT present in the buffer substrate during incubation. In the determinations, which were performed in quadruplicate, the concentration of labeled 5-HT was maintained constant (8×10^{-4} M, 140 μg/ml). Enzymatic activity was calculated from the known specific activity of the substrate after subtracting the corresponding blanks.

The significance of results was determined by Student's t test and the correlation and linear regression analyses were performed according to current statistical procedures.

RESULTS. *Electron microscopic cytochemistry.* The adrenergic nerve endings of the normal vas deferens exhibit a positive glutaraldehyde-dichromate (GD) reaction, and the prefixation with formaldehyde results in a negative picture (FGD) (fig. 1A). This confirms that a catecholamine (most probably NE) is responsible for the reaction observed with the GD procedure and indicates that 5-HT is not present within the adrenergic nerve vesicles, at least in cytochemically detectable quantities (Jaim-Etcheverry and Zieher, 1968b). After incubation of vas deferens slices with increasing concentrations of 5-HT,

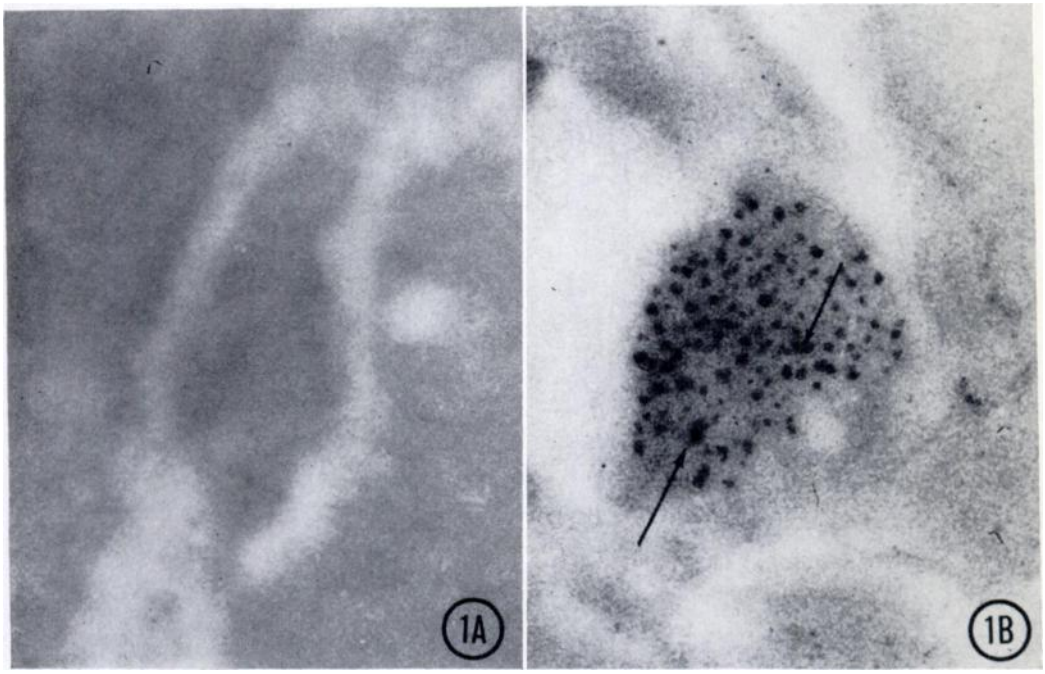

FIG. 1. Electron micrographs of sympathetic nerves between smooth muscle cells in rat vas deferens processed with the cytochemical technique for the demonstration of 5-HT (FGD reaction: formaldehyde fixation and glutaraldehyde-potassium dichromate treatment). The nerve in A corresponds to a normal rat and therefore no reactive sites are observed. The terminal in B corresponds to a slice incubated for 40 minutes in 5-HT, 5.6×10^{-4} M (100 μg/ml). Two types of reactive sites may be distinguished by their size: small granules of 200 to 300 Å in diameter and larger dense precipitates of 500 to 600 Å (arrows). These densities can be matched with the dense cores of the small and large granulated vesicles observed with conventional techniques in these endings. ×60,000.

TABLE 1

Concentration of endogenous norepinephrine and of 5-hydroxytryptamine accumulated in vas deferens slices[a]

Results are expressed in micrograms per gram of wet weight as mean values ± S.E. for six experiments and as percent decrease in the case of norepinephrine concentration.

5-HT Conc. in Medium		5-Hydroxytryptamine	Norepinephrine	
M	*μg/ml*	*μg/g*	*μg/g*	%
None		—[b]	18.82 ± 1.08	
5.6×10^{-5}	10	2.02 ± 0.23	18.37 ± 1.18	
2.8×10^{-4}	50	3.75 ± 0.73	17.49 ± 0.79	7
5.6×10^{-4}	100	4.23 ± 0.53	15.86 ± 0.96[c]	16
1.7×10^{-3}	300	7.86 ± 1.09	15.17 ± 0.88[d]	19
2.8×10^{-3}	500	10.19 ± 0.90	14.19 ± 0.50[e]	25
4.5×10^{-3}	800	16.82 ± 0.81	14.15 ± 0.83[e]	25
5.6×10^{-3}	1000	20.98 ± 1.49	12.43 ± 1.34[f]	34

[a] Vas deferens slices were incubated for 15 minutes at 37°C in McEwen's saline, 40 minutes in medium plus different concentrations of exogenous 5-HT and washed for a further 15 minutes in saline without additions.

[b] Endogenous 5-HT was undetectable in slices incubated in medium without 5-HT.

[c] P between .1 and .05.

[d] $P < .05$.

[e] $P < .01$.

[f] $P < .005$.

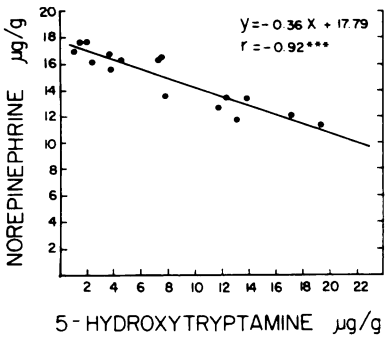

FIG. 2. Correlation between the increase in concentration of 5-HT and the decrease of endogenous NE in vas deferens slices incubated in the presence of various concentrations of 5-HT (10, 100 and 1000 μg/ml). The regression line corresponding to these observations is also shown. ***, $P < .001$.

the reactive pattern observed with the FGD reaction changes. No variations are apparent from the cytochemical characteristics of normal vas deferens when the incubation is performed in the lower concentrations used ($<2.8 \times 10^{-4}$ M). A positive FGD reaction appears in the adrenergic nerve vesicles when the concentration of exogenous 5-HT is 5.6×10^{-4} M or higher (fig. 1B). As stated above, the characteristics of the material processed with the cytochemical reactions make difficult any quantitative study. For this reason, no attempt was made to determine precisely the concentration of 5-HT in the medium which results in the first appearance of reactive sites in the nerves, but this concentration is somewhere between 2.8×10^{-4} and 5.6×10^{-4} M. The FGD reaction indicates that the exogenous amine is stored in cytochemically detectable amounts within nerve vesicles. Small (300–500 Å in diameter) as well as large (700–900 Å) reactive sites are observed in the nerves, thus confirming the participation of both types of vesicles in the amine storage process (see Jaim-Etcheverry and Zieher, 1969b).

Biochemical correlates. The content of NE and 5-HT in slices incubated in different concentrations of 5-HT was assayed at the end of the incubation. The results are shown in table 1. 5-HT, which is undetectable in control slices, markedly increases in the tissue as a function of its concentration in the incubation medium. On the other hand, NE shows a dose-dependent decrease. The 16% diminution in the NE content of slices incubated with 5-HT, 5.6×10^{-4} M, is at the limit of significance when compared with control values. With higher concentrations of 5-HT, significant decreases of 19 to 34% are observed depending on the concentration of 5-HT used for incubation. This depletion increased to 50% when concentrations of 5-HT higher than those reported here were used for incubation. A

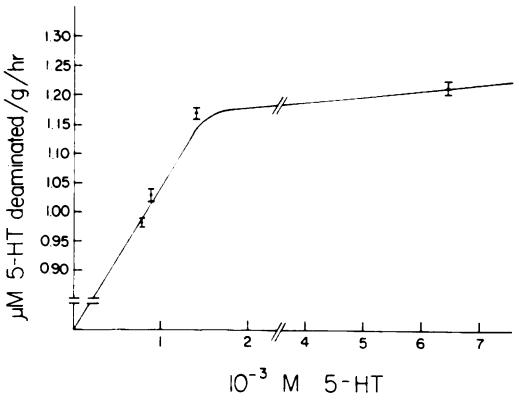

FIG. 3. Effect of various concentrations of 5-HT on the MAO activity of vas deferens slices. The incubation was performed in a solution containing 0.8 mM ^{14}C-5-hydroxytryptamine in 0.1 M potassium phosphate buffer, pH 7.2, for 30 minutes at 37°C. Unlabeled 5-HT was added to the medium to achieve the final molarities expressed. Points represent mean values ± S.E. (brackets) of four determinations, each performed in quadruplicate.

highly significant correlation between the increase of 5-HT and the decrease of NE was found in incubated slices. The points representing the observed values fit into the regression line shown in figure 2 calculated by the method of least squares.

The rate of 5-HT metabolism in vas deferens slices, a function of 5-HT concentration, is shown in figure 3. It may be observed that the enzyme monoamine oxidase is saturated when concentrations of 5-HT higher than 5.6×10^{-4} M are present in the medium. These results are in agreement with those obtained for brain MAO (McCaman *et al.*, 1965).

Cell fractionation. The concentrations of endogenous NE and protein in the different fractions obtained from the normal vas deferens are shown in table 2. The results agree with previous reports of Jarrot and Iversen (1968) and Thoa *et al.* (1969). The occurrence of NE in all fractions is explained by results of fluorescence histochemical studies (Jonsson and Sachs, 1969) which have shown, in mouse atrium homogenates, nonhomogenized nerves in the coarse fraction. In the mitochondrial fraction a few fluorescent dots, probably corresponding to nondisrupted varicosities, were present while the microsomal fraction showed an even and intense

TABLE 2

Distribution of endogenous norepinephrine and of 5-hydroxytryptamine accumulated in the different subcellular fractions from vas deferens slices[a]

Results are expressed in micrograms per gram of wet weight as mean values ± S.E. for four to six experiments, as percent recovered and as relative specific concentration (RSC[b]) of both amines in the different fractions.

5-HT Conc. in Medium	Fraction	Norepinephrine	%	RSC	5-Hydroxytryptamine	%	RSC
M		μg/g			μg/g		
None	Coarse	3.66 ± 0.73	32.1	0.96			
	Mitochondrial	1.81 ± 0.42	15.9	0.68			
	Microsomal	3.01 ± 0.32	26.4	4.53			
	Supernatant	2.91 ± 0.30	25.5	0.69			
	Total	11.41					
5.6×10^{-5}	Coarse	3.12 ± 0.47	29.3	0.88	0.326 ± 0.038	25.3	0.75
	Mitochondrial	1.62 ± 0.38	15.2	0.65	0.165 ± 0.032	12.8	0.55
	Microsomal	2.92 ± 0.24	27.5	4.71	0.447 ± 0.071	34.7	5.95
	Supernatant	2.96 ± 0.46	27.8	0.75	0.350 ± 0.031	27.2	0.73
	Total	10.63			1.288		
5.6×10^{-4}	Coarse	2.41 ± 0.31	31.1	0.99	0.689 ± 0.185	26.1	0.78
	Mitochondrial	1.15 ± 0.15	14.8	0.63	0.395 ± 0.060	14.9	0.64
	Microsomal	2.11 ± 0.26	27.2	4.68	0.818 ± 0.139	31.0	5.33
	Supernatant	2.97 ± 0.34	26.7	0.72	0.734 ± 0.073	27.8	0.75
	Total	7.75			2.636		
5.6×10^{-3}	Coarse	2.29 ± 0.27	36.4	1.09	2.393 ± 0.405	17.7	0.53
	Mitochondrial	0.69 ± 0.10	10.9	0.47	1.522 ± 0.088	11.3	0.48
	Microsomal	1.80 ± 0.09	28.6	4.92	1.890 ± 0.214	14.0	2.41
	Supernatant	1.49 ± 0.11	23.7	0.64	7.663 ± 0.266	56.8	1.53
	Total	6.28			13.468		

[a] Vas deferens slices were incubated for 15 minutes at 37°C in McEwen's saline, 40 minutes in medium plus different concentrations of exogenous 5-HT and washed for a further 15 minutes in saline without additions.

[b] RSC is the relative specific concentration of NE and 5-HT and corresponds to the ratio percent amine recovered/percent protein recovered. The total protein content of vas deferens homogenate was 62.02 ± 5.68 mg/g, distributed as follows: 33.5% in the coarse fraction, 23.5% in the mitochondrial fraction, 5.9% in the microsomal fraction and 37.2% in the supernatant.

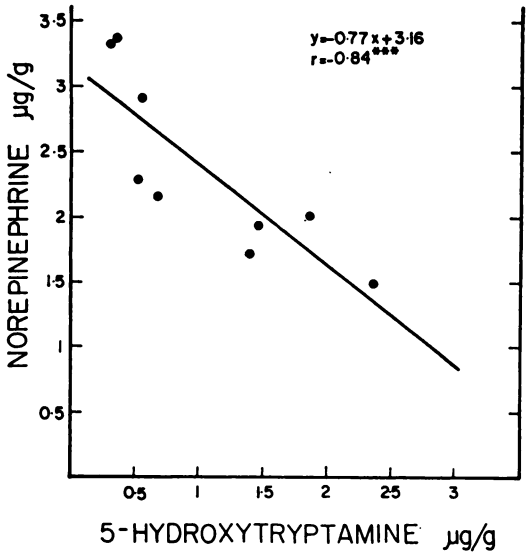

FIG. 4. Correlation between the increase in 5-HT and the decrease of NE concentration in the microsomal fraction of vas deferens slices incubated in the presence of exogenous 5-HT, its concentration ranging from 5.6×10^{-6} to 5.6×10^{-3} M (10–1000 µg/ml). The regression line corresponding to these observations is also shown. ***, $P < .001$.

TABLE 3

Ratios between concentrations of 5-hydroxytryptamine and norepinephrine in intact vas deferens slices, in the total particulate and in the microsomal fraction obtained from incubated slices[a]

	5-HT/NE Ratios at 5-HT Conc. in Medium of:		
	5.6×10^{-6} M	5.6×10^{-4} M	5.6×10^{-3} M
Intact slices	0.110	0.264	1.532
Total particulate	0.122	0.337	1.214
Microsomal fraction	0.153	0.386	1.047

[a] Vas deferens slices were incubated for 15 minutes at 37°C in McEwen's saline, 40 minutes in medium plus different concentrations of exogenous 5-HT and washed for a further 15 minutes in saline without additions.

fluorescence. In their studies the mitochondrial and the microsomal fraction were centrifuged and analyzed together (fraction P_2 of Jonsson and Sachs, 1969) because of their similar amine-uptake characteristics. However, the relative specific concentration (RSC) of NE (percent NE recovered/percent protein recovered) is much greater in the microsomal fraction. This indicates that the amine storage vesicles are recovered in a purer form in the latter fraction and is the reason why both fractions have been analyzed here separately.

Table 2 also shows the distribution of NE and 5-HT in the different fractions obtained from slices incubated in three different concentrations of exogenous 5-HT. The RSC of endogenous NE in the different fractions remains unchanged although there is a diminution of total NE depending on the concentration of exogenous 5-HT. This indicates that the amine is evenly depleted from all the fractions. The subcellular distribution of 5-HT accumulated in the tissue roughly parallels that of the endogenous NE although an even higher percentage of 5-HT is recovered in the microsomal fraction. This microsomal peak is found with 5.6×10^{-6} and 5.6×10^{-4} M 5-HT in the medium, whereas at 5×10^{-3} M a large amount of the 5-HT taken up is recovered in the supernatant. This causes a diminution of the RSC of 5-HT in the microsomal fraction with a corresponding increase in the high-speed supernatant. The increase in 5-HT in the microsomal fraction is significantly correlated with a decrease of NE, and the regression line obtained by the method of least squares for this relationship is shown in figure 4.

The ratios between the 5-HT and NE concentrations in the tissue after incubation of the slices in 5-HT are shown in table 3. Ratios corresponding to intact slices, to total particulate (resulting from the addition of coarse, mitochondrial and microsomal fractions) and to the microsomal fractions are compared. In 5.6×10^{-6} or 5.6×10^{-4} M 5-HT the relative amount of 5-HT present in the total particulate and in the microsomal fraction is proportionately higher than that of NE present in the same fractions. The changes observed when the tissue is incubated in 5.6×10^{-3} M are most probably due to the presence of a higher amount of 5-HT in the supernatant which reduces the ratios 5-HT/NE in the particulate fractions when compared to the total homogenate.

Accumulation of 5-HT in chemically denervated slices. As shown in figure 5, total NE is depleted by 84% after the injection of 6-OHDA (two 68 mg/kg i.v. doses) and the amount of amine recovered in the microsomal fraction is decreased to aproximately the same extent. After incubation of slices from 6-OHDA-treated rats in 5-HT, there are no significant variations in

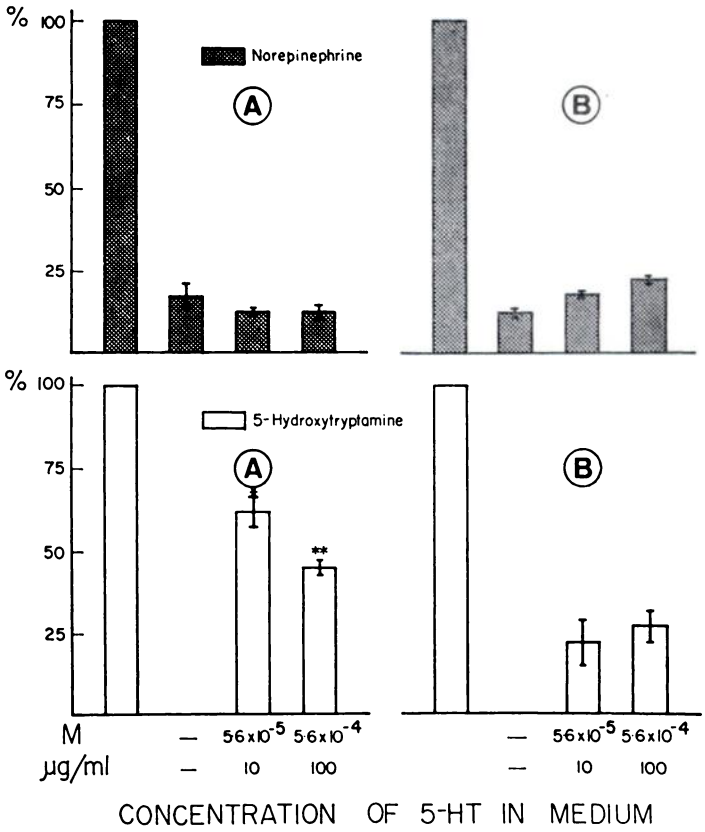

FIG. 5. Effects of 6-OHDA, 68 mg/kg i.v., 96 and 48 hours before killing on the content of endogenous NE and on 5-HT accumulation in vas deferens slices incubated with exogenous 5-HT, 5.6×10^{-4} and 5.6×10^{-5} M (10 and 100 µg/ml). Experimental values are expressed as the percent reduction when compared with the corresponding values obtained in nondenervated slices. Values found in intact slices are given in A and those of the microsomal fraction in B. Endogenous 5-HT could neither be detected in total slices nor in the microsomal fraction. The columns represent mean values ± S.E. (brackets) of four to five experiments. *, $P < .05$; **, $P < .01$; differences between the rest of the values and corresponding controls, $P < .001$.

the NE content of the slices or in the amount recovered in the microsomal fraction. The 5-HT accumulated in vas deferens slices denervated by 6-OHDA is only 61 and 45%, respectively of that found when nondenervated slices are incubated in the same conditions. In fractions from denervated slices incubated with 5-HT, 5.6×10^{-5} and 5.6×10^{-4} M, it was found that the 5-HT present in the microsomal fraction is 25% of that recovered in the same fraction from nondenervated slices incubated in the same conditions.

DISCUSSION. Evidence obtained from fluorescence histochemical, electron microscopic as well as pharmacologic and biochemical studies has shown the capacity of peripheral adrenergic nerves to incorporate 5-HT (Owman, 1964; Taxi and Droz, 1966; Snipes *et al.*, 1968; Eccleston *et al.*, 1968; Jaim-Etcheverry and Zieher, 1969a). The ultrastructural observations reported here demonstrate that in slices of vas deferens incubated in a medium containing 5-HT it is possible to detect cytochemically the presence of the exogenous amine within adrenergic nerve vesicles without depleting their endogenous NE. This morphologic demonstration is achieved only when high concentrations of 5-HT are present in the vicinity of the terminal. This confirms that the failure to demonstrate reactive cores in vas deferens nerves after injection of high doses of 5-HT combined with MAO inhibitors is due to the fact that the concentration of 5-HT present in the vesicles is not enough to be revealed with the cytochemical procedure (Jaim-Etche-

verry and Zieher, 1969a). The endogenous NE renders difficult the incorporation of sufficient 5-HT to be cytochemically detectable since in vesicles depleted of their NE, 5-HT is readily demonstrable both *in vivo* and *in vitro* (Jaim-Etcheverry and Zieher, 1969a; Snipes *et al.*, 1968). The aim of the present study was to analyze the adrenergic nerve vesicles in conditions in which 5-HT may be detected cytochemically. Since high concentrations of the amine need to be present in the incubation medium to achieve this accumulation, no conclusions can be drawn on the characteristics of the process of 5-HT incorporation by the adrenergic neuron. The mechanism of this uptake has been recently studied with an appropriate experimental design and found to be a temperature-dependent and saturable process most probably similar to that responsible for NE uptake (Eccleston *et al.*, 1968; Thoa *et al.*, 1969). The experimental system used roughly reproduces the conditions found in the normal pineal gland of the rat where nerve endings are exposed to very high concentrations of 5-HT. This concentration must be at least of a similar magnitude to the one which results in the cytochemically reactive cores in the system *in vitro* (Jaim-Etcheverry and Zieher, 1971).

It is possible to draw some conclusions regarding the fate of endogenous NE in the conditions in which 5-HT is known to be present within adrenergic nerve vesicles. On the basis of observations of electron micrographs of pineal nerve endings, we have proposed that NE and 5-HT probably coexist in the same adrenergic nerve vesicle (Jaim-Etcheverry and Zieher, 1968b). However, it is impossible to conclude this purely on cytochemical grounds. The analysis of experiments *in vitro* has given more precise information on this point. If the 5-HT incorporated into adrenergic vesicles causes a displacement of the intravesicular NE, one might expect a reduction in the total NE content since the extravesicular NE would be metabolized. However, with the concentration which results in the cytochemical detection of 5-HT within the majority of the vesicles and endings, there is only a slight decrease of NE. This might be due to an extravesicular accumulation of the displaced amine. This possibility is ruled out by subcellular distribution studies because the percentage of NE found in the particulate and in the supernatant is similar to that in control slices. As discussed below, 5-HT is also concentrated in particulate fractions. This suggests that both amines are present at the same time in the storage organelles and indicates the existence of a certain availability of storage sites in adrenergic nerve vesicles even under normal conditions (von Euler, 1966; Stjärne, 1966). A similar conclusion is drawn from experiments in which the incubation of slices in higher concentrations of 5-HT (5.6×10^{-3} M) significantly depletes NE. Again in this situation, the relative specific concentration of NE in all fractions is unchanged with respect to that in control slices. The fact that the vesicular NE displaced is either metabolized or released from the tissue is indicated by the lack of accumulation of NE in the supernatant. Since MAO has less affinity for NE than for 5-HT (Pletscher *et al.*, 1966), the saturation of MAO found with concentrations of 5-HT in the medium higher than 5.6×10^{-4} M indicates that in these conditions, the vesicular NE displaced is most probably not attacked by MAO and therefore leaves the endings unchanged or is destroyed along other metabolic pathways. Moreover, 5-HT at high concentrations has a dual action because it releases NE and simultaneously inhibits reuptake of the liberated catecholamine (see Iversen, 1967; Burgen and Iversen, 1965). This situation might also account for the tendency of the RSC of NE in the supernatant to decrease when the incubation is performed with increasing concentrations of 5-HT.

The fact that MAO activity of vas deferens slices is saturated at concentrations of 5-HT higher than 5.6×10^{-4} M suggests that the nonlinearity of 5-HT accumulation in intact slices with lower concentrations of exogenous 5-HT is due to the enzymatic destruction of the amine. Most probably the high concentrations of exogenous 5-HT necessary to allow its detection in nerve vesicles are in part due to the enzymatic destruction of 5-HT which occurs at concentrations lower than 5.6×10^{-4} M. Once the enzyme is saturated, the unmetabolized 5-HT can enter the vesicles.

The endogenous as well as the exogenous incorporated NE in sympathetically innervated organs is highly concentrated in the microsomal fraction (Potter and Axelrod, 1962). It is in this fraction that the amine storage vesicles are

considered to sediment (see Potter, 1966, 1967). Recent studies have shown a similar concentration in the microsomal fraction of exogenous 5-HT accumulated in guinea-pig vas deferens (Thoa *et al.*, 1969). This finding has been confirmed since the exogenous 5-HT accumulated in vas deferens slices parallels the distribution of endogenous NE, and a high percentage is found in the microsomal fraction where its relative specific concentration is even higher than that of NE. The coexistence of both NE and 5-HT within the same storage compartment is further supported by the similarity of concentration ratios in the intact slices as well as in the total particulate and microsomal fractions. Moreover, the correlation between 5-HT increase and NE depletion found in intact slices is also found in the microsomal fraction. The amount of NE displaced from the organelles in this fraction per unit increase in 5-HT concentration is greater than in intact slices as shown by the slopes of the regression lines. This indicates a more selective depletion of NE from the storage organelles. From these experiments it seems that adrenergic nerve vesicles have in normal conditions a certain capacity for storing exogenous 5-HT without a significant alteration of their NE content. However, when more 5-HT is forced into the vesicles, NE is displaced. This finding and results from experiments performed in the nerves of the rat pineal gland (Jaim-Etcheverry and Zieher, 1971) suggest that both amines share a similar site in the vesicular storage complex.

Apart from the presence of 5-HT in the microsomal fraction of vas deferens homogenates, the fact that neural structures are partly responsible for its accumulation is confirmed by the experiments performed on slices from animals treated with 6-OHDA. The depletion of NE found in intact slices and in the microsomal fraction is in agreement with results of previous experiments in which this decrease has been found to reflect a selective destruction of adrenergic endings (Tranzer and Thoenen, 1968; Thoenen and Tranzer, 1968). The 50% reduction in the 5-HT present in the total homogenate after incubation of denervated slices indicates the participation of adrenergic endings in its accumulation. Moreover, the fact that the 5-HT recovered in the microsomal fraction after incubation is 75% less than in nondenervated slices confirms that the accumulation of 5-HT is into the amine storage vesicles which disintegrate after sympathectomy. The greater reduction observed in the microsomal fraction when compared to the total homogenate also indicates the existence of an important extravesicular accumulation of the amine. This extramicrosomal 5-HT in denervated slices may reflect its non-neuronal accumulation, which is to be expected in view of the very high concentrations of 5-HT used for incubation. A similar situation exists with regard to NE uptake in denervated organs (see Iversen, 1967; Jonsson *et al.*, 1969). Also, mast cells are present in the outer coat of the vas deferens and probably contribute to the extraneuronal uptake since in the rat they store 5-HT. Alternatively, 5-HT may be incorporated in the axoplasm of preterminal nerve trunks which are apparent as "strongly fluorescent bulges" consecutive to destruction of the endings by 6-OHDA (Malmfors and Sachs, 1968). The origin of the microsomal 5-HT in denervated slices is difficult to determine. A microsomal although nonvesicular localization of the incorporated amine might be postulated, but most probably 5-HT is taken up in vesicles in nerves which have escaped destruction by the drug or in vesicles which are supposed to accumulate in preterminal nerve trunks after 6-OHDA treatment, since these are the sites in which residual NE is most probably localized (Thoenen and Tranzer, 1968; Malmfors and Sachs, 1968).

This "common vesicular storage" mechanism, which is cytochemically and biochemically detectable, is present under normal conditions in certain adrenergic nerves such as those of the pineal gland of the rat, or it may be detected by the administration of substances acting as false adrenergic neurotransmitters. Most probably, it is also present in synaptic vesicles of other peripheral and central nerve endings, although the chemical nature or concentration of substances stored may prevent its cytochemical or biochemical demonstration. This mechanism of coexistence of more than one active substance in a nerve ending and more precisely within synaptic vesicles, might prove to be of great physiologic or pharmacologic importance.

CONCLUSIONS. When exogenous 5-HT is incorporated into adrenergic nerve endings of the vas deferens *in vitro* and becomes cytochemically detectable, it coexists with endogenous NE

in the nerve storage vesicles. If the concentration of exogenous and therefore of vesicular 5-HT further increases, the endogenous NE is gradually displaced from its stores as shown by the significant correlation found between these phenomena in both incubated vas deferens slices and in the microsomal fraction derived from them. Similar experiments performed on tissues chemically denervated by 6-OHDA confirm the participation of adrenergic nerve endings in the 5-HT accumulation process.

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