THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 198, No. 3

*Printed in U.S.A.*

# SEROTONIN ACCUMULATION IN THE GUINEA-PIG MYENTERIC PLEXUS: ION DEPENDENCE, STRUCTURE-ACTIVITY RELATIONSHIP AND THE EFFECT OF DRUGS[1]

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Accepted for publication April 20, 1976

## ABSTRACT

GERSHON, MICHAEL D., ROBERT G. ROBINSON AND LEONARD L. ROSS: Serotonin accumulation in the guinea-pig myenteric plexus: Ion dependence, structure-activity relationship and the effect of drugs. J. Pharmacol. Exp. Ther. **198:** 548–561, 1976.

The specific accumulation of serotonin in neurons of the guinea-pig myenteric plexus was examined. Nonadrenergic neurons have been shown to be responsible for this accumulation. Serotonin accumulation, known to be sodium-dependent, was found to be inhibited by elevating the external potassium concentration. Accumulation was inversely related to the concentration of potassium and was also decreased in the presence of 0 mM $Ca^{++}$ or 12 mM $Ca^{++}$. The affinity of analogous molecules for the myenteric plexus was greatly reduced in compounds which had no alkyl amino side chain, in which the amino group was methylated or which had no 5-hydroxyl group. Most analogs competitively inhibited serotonin accumulation and 6-hydroxytryptamine was demonstrated by histofluorescence to be taken up into the myenteric plexus after chemical sympathectomy with 6-hydroxydopamine. Serotonin accumulation was also inhibited by tricyclic antidepressants and amphetamines. The inhibition of serotonin accumulation by these compounds differed from their inhibition of accumulation of norepinephrine. As in the central nervous system, chlorimipramine was the most potent tricyclic antidepressant against accumulation of serotonin while desmethylimipramine was the most potent inhibitor of the accumulation of norepinephrine. Amphetamines were more effective inhibitors of serotonin accumulation than were tricyclic antidepressants, but all of these drugs were more effective against the accumulation of norepinephrine than serotonin. This study confirms the existence of a unique population of axons in the mammalian myenteric plexus which are distinguishable by their characteristic accumulation of serotonin and have not been found elsewhere in the peripheral nervous system.

Received for publication July 7, 1975.
[1] Supported by Public Health Service Grants NS07436 and NS11364.

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5-Hydroxytryptamine (serotonin, 5-HT) may be a neurotransmitter in the mammalian myenteric plexus (Bülbring and Gershon, 1967). A number of lines of evidence support this hypothesis: 5-HT activates both excitatory and inhibitory ganglion cells in the plexus (Gaddum, 1953; Gaddum and Picarelli, 1957; Brownlee and Johnson, 1963; Gershon, 1967; Drakontides and Gershon, 1968; Dingledine *et al.*, 1974; Takayanagi *et al.*, 1974); it is released from the wall of the gut when intestinal neurons are stimulated (Bülbring and Gershon, 1967); and drugs which antagonize responses to 5-HT interfere with vagal relaxation of the stomach (Bülbring and Gershon, 1967). Recently it has been shown that chronic treatment of guinea pigs with morphine or *p*-chlorophenylalanine, an inhibitor of the rate-limiting enzyme in the biosynthesis of 5-HT, causes the intestine to become supersensitive to the amine (Schulz and Goldstein, 1973, 1974). These latter observations are suggestive of the development of denervation supersensitivity to 5-HT. The intestine also has an adequate mechanism for the inactivation of the putative transmitter. Neurons of the myenteric plexus actively take up 5-HT (Robinson and Gershon, 1971; Gershon and Altman, 1971). These neurons have a high affinity for 5-HT and are not adrenergic although at high concentrations of 5-HT there is also a nonspecific uptake of that amine into adrenergic axon terminals (Robinson and Gershon, 1971; Drakontides and Gershon, 1972).

A role for 5-HT as a neurotransmitter in the myenteric plexus has not been universally accepted. One major problem is that the amine is difficult to demonstrate as an endogenous constituent of the myenteric plexus in mammals (Dubois and Jacobowitz, 1974), although it has been found in relatively large amounts in the neurons of the intestine of cyclostomes (Baumgarten *et al.*, 1973). Histochemical studies in mammals have been conflicting. Evidence supporting the endogenous synthesis of 5-HT in the mammalian plexus has been obtained from examination of whole mounts of the dissected longitudinal muscle and adherent myenteric plexus of guinea-pig ileum (Robinson and Gershon, 1971). There is an increase in the concentration of 5-HT and a formaldehyde-induced fluorophore appears in this preparation following inhibition of monoamine oxidase. Others have been unable to detect 5-HT in the rat or guinea-pig myenteric plexus histochemically in frozen dried paraffin sections despite the administration of tryptophan to rats (Dubois and Jacobowitz, 1974) or reserpine and pheniprazine to guinea pigs (Ahlman and Enerbäck, 1974).

The present investigation was undertaken to characterize further the accumulation of 5-HT in nonadrenergic neurons of the guinea-pig myenteric plexus. The study was also done to assess the usefulness of the myenteric plexus as a peripheral model for the study of serotonergic neurons. The uptake of 5-HT has already been shown to have the following characteristics: uptake is linear for at least 30 minutes; the $K_m$ is $7.4 \times 10^{-7}$ M and the $V_{max}$ is 0.22 nmol/g/min; uptake is highly temperature dependent, the $Q_{10}$ (27–30°C) is 3.6; uptake is reduced by inhibitors of glycolysis but not by inhibitors of aerobic metabolism; uptake is antagonized by ouabain and is sodium dependent; and uptake is not inhibited by norepinephrine even when that substance is present at 10 times the concentration of 5-HT (Gershon and Altman, 1971).

## Methods

Male albino English short hair guinea pigs (300–350 g) were stunned by a blow on the head and exsanguinated. The small intestine was stripped from its mesenteric attachment and placed in Krebs' solution which had previously been gassed with 95% $O_2$–5% $CO_2$ at 24°C. The longitudinal muscle layer and adherent myenteric plexus were dissected from the gut and cut into strips about 1 cm in length.

Strips were incubated with continuous agitation in 10 ml of Krebs' solution at 37°C under an atmosphere of 95% $O_2$–5% $CO_2$. In initial experiments involving the addition of tritiated DL-norepinephrine ($^3$H-NE), ascorbic acid, 0.2 mg/ml, and disodium ethylenediamine tetraacetic acid (EDTA), 0.05 mg/ml, were added to the Krebs' solution. However, these compounds were no longer added when it was found that they did not detectably alter the measured uptake of $^3$H-NE. Tissues were allowed to equilibrate at 37°C for 30 minutes before $^3$H-NE (9.95 Ci/mmol, New England Nuclear Corp, Boston, Mass.) or $^3$H-5-HT (3.0 Ci/mmol, Amersham Searle, Des Plaines, Ill.) was added to the incubation medium. Strips were incubated with the tritiated monoamine for 20 minutes. Experimental compounds were present in the incubating media throughout both the equilibration period and the time of incubation with $^3$H-5-HT or $^3$H-NE.

Following incubation, each strip was transferred to a separate vial containing 2.0 ml of oxygenated Krebs' solution at 0–4°C. Tissue strips were then transferred

at regular time intervals through a series of vials, each containing 2.0 ml of fresh, oxygenated Krebs' solution. The tissues were thus washed by serial transfer at 0–4°C for a total of 2 hours. Vials were continuously agitated throughout the washout procedure. In each vial, the concentration of radioactivity in tissue was much greater than in the Krebs' solution. Thus efflux of the tritiated monoamines was promoted and little or no influx of radioactive material should have occurred during the washout. Following washout, strips were carefully blotted, weighed and placed overnight in vials containing 0.6 ml of 0.1 M HCl and 1.4 ml of 70% ethanol. This solution extracts essentially all of the radioactivity remaining in the strips (Fischman and Gershon, 1964; Gershon and Altman, 1971). The radioactivity in each of the vials was determined by liquid scintillation counting. A dioxane-based cocktail was used containing ethylene glycol monoethyl ether (200 ml/l), naphthalene (60 g/l) and Omnifluor (8 g/l, New England Nuclear). Samples were corrected for quench by external standardization.

Washout curves were plotted for each tissue. The percentage of the initial radioactivity (sum of radioactivity washed out of the tissue and that extracted after washout) remaining in the tissue was plotted semilogarithmically as a function of time. The resulting curves are not linear (Gershon and Altman, 1971). However, after 30 minutes of washout, the logarithmic rate of tritium efflux does approach a straight line and can be described by the single equation $Q_t = Q_0^{-\lambda t}$ where $Q_0$ is the initial amount of material present in the compartment represented by the equation; and $\lambda$ is the rate constant of washout. $Q_0$ and $\lambda$ were determined from the curves with the aid of a computer. Over 95% of the radioactivity present in this compartment after 30 minutes of washout migrated with authentic 5-HT (or NE when $^3$H-NE was used) on thin-layer chromatograms. Radioautographic examination of tissue fixed after washout revealed that essentially all of the remaining radioactivity is confined to the myenteric plexus (Gershon and Altman, 1971). Therefore, the labeled material washing out of strips during the first 30 minutes of washout appeared to be derived from several compartments with varying rate constants. However, after this time, only a single compartment that appeared to represent $^3$H-5-HT or $^3$H-NE in the myenteric plexus and had a single slow rate of efflux seemed to be involved. For this reason only $Q_0$, calculated as described, was used and taken to represent the specific accumulation of 5-HT or NE in each experiment. Since accumulation is linear for at least 30 minutes, accumulation of $^3$H-5-HT during the 20-minute incubation period used in these experiments reflects the initial rate of uptake of the amine.

For the histochemical demonstration of monoamines, whole mounts of dissected longitudinal muscle and myenteric plexus were prepared as described previously (Robinson and Gershon, 1971). Essentially, tissues were stretched onto glass slides with the myenteric plexus facing up. The slides were dried *in vacuo* over phosphorus pentoxide for 12 to 24 hours. After drying, the slides were exposed at 80°C to formaldehyde gas generated first from paraformaldehyde previously equilibrated at 70% relative humidity and then again from paraformaldehyde equilibrated at 90% relative humidity (Fuxe and Jonsson, 1967). After treatment with formaldehyde, slides were mounted in nonfluorescent immersion oil (Leitz) and examined by fluorescence microscopy.

Except where otherwise stated, standard statistical methods were used (Goldstein, 1965). All regression lines were fitted by the method of least squares. Four concentrations of each inhibitor were tested and at least four experiments were done at each concentration. The ID50, the concentration of each inhibitor which reduced amine accumulation to 50% of the control, was obtained by log-probit analysis; 95% confidence limits for the ID50 were also calculated. The method of Hofstee (1952) was used to determine the maximum rate of amine uptake ($V_{max}$) as well as the concentration of amine at which the rate of uptake is half of the maximum rate ($K_m$). The concentration of competitive inhibitors which reduced the velocity of amine accumulation to one-half the rate shown in the absence of the inhibitor ($K_i$) was estimated by the method of Dixon (1953).

## Results

**Ionic requirements for the accumulation of 5-HT.** Dependence of the uptake of 5-HT on the presence of ionic sodium in the incubating medium has already been demonstrated (Gershon and Altman, 1971). Ion concentrations in the incubation medium were varied in order to determine the importance for 5-HT accumulation of the cations $K^+$ and $Ca^{++}$, and the $Cl^-$ anion. The results of these experiments are shown in figure 1 and table 1. In experiments in which the potassium concentration was raised, $K_2SO_4$ was added to the Krebs' solution. $Na_2SO_4$ was added appropriately to the incubating medium of controls to match the osmolarity of the incubation solutions. Note that $SO_4^=$ does not appear to interfere with accumulation of 5-HT by the myenteric plexus since replacement of NaCl by $Na_2SO_4$ (compare 1 with 4 and 5 in table 1) had no effect on the accumulation. In those experiments in which the calcium concentration was increased (table 1, solution 3), $NaHCO_3$ and $NaH_2PO_4$ were omitted from the solution, Tris was used as the buffer and tissues were gassed with 100% $O_2$ instead of 95% $O_2$ and

TABLE 1

*Effect of changes in the ionic composition of the incubation medium on the accumulation of 5-HT by the myenteric plexus*

| Solution | No. of Tissues | Accumulation[a] | Ratio Expt/-Control[b] |
|---|---|---|---|
| | | *ml/g* | |
| 1. Normal Krebs' | 6 | 6.3 (±0.3) | |
| 2. 0 mM $Ca^{++}$ | 5 | 3.1 (±0.1)[c] | 0.49 |
| 3. 12.5 mM $Ca^{++}$ | 5 | 2.6 (±0.2)[c] | 0.41 |
| 4. 94 mM $Na_2SO_4$; 2.5 mM $K_2SO_4$ (no chloride) | 5 | 5.9 (±0.7)[d] | 0.94 |
| 5. Normal Krebs' with 37.5 mM $Na_2SO_4$ (control for osmolarity) | 6 | 5.2 (±0.8)[d] | 0.83 |
| 6. Krebs' with Tris buffer no $NaHCO_3$ no $Na_2HPO_4$ gassed with 100% $O_2$ | 6 | 6.3 (±0.3)[d] | 1.00 |

[a] Accumulation = dpm/g of tissue in the compartment represented by the slowest single exponential component of the washout curve divided by dpm/ml of 5-HT in the incubating solution at the end of the incubation period. This tissue/medium ratio constitutes a 5-HT space (±S.E.).

[b] 5-HT space of experimental tissue divided by 5-HT space of the control.

[c] $P < .001$ when compared to group 6.

[d] P is not significant when compared to group 1.

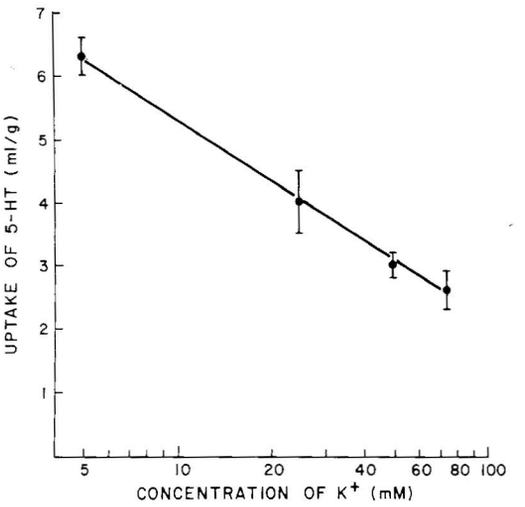



FIG. 1. Accumulation of serotonin (disintegrations per minute per gram of tissue ÷ disintegrations per minute per milliliter of incubating medium or milliliters per gram) is plotted as a function of the logarithm of the concentration of potassium in the incubating medium. Accumulation in the presence of elevated potassium is significantly less than control (5 mM): for 25 mM potassium, $P < .02$; for 50 and 75 mM potassium, $P < .001$. Serotonin accumulation at 75 mM potassium is significantly less than that at 25 mM, $P < .05$. The mean ± S.E. accumulation of serotonin in five to six tissues is plotted at each concentration of potassium.

5% $CO_2$. Accumulation of 5-HT in these experimental tissues was compared to controls (table 1, solution 6) incubated in a similar solution but with a normal concentration of $Ca^{++}$. EDTA (0.1 mg/ml) was added to solutions meant to contain no calcium.

Accumulation of 5-HT by the myenteric plexus was inhibited by increasing the concentration of $K^+$ in the medium (fig. 1). The decrease in the accumulation of 5-HT was related to the log of the concentration of $K^+$ (fig. 1). Accumulation was also inhibited when tissues were incubated in the presence of either no $Ca^{++}$ (table 1, solution 5) or excess (12.5 mM) $Ca^{++}$. On the other hand, accumulation of 5-HT appeared to be relatively independent of anions in that $SO_4^=$ could substitute for $Cl^-$.

**Inhibition of 5-HT uptake by related amines: Effects of N-substitutions.** In order to determine the significance of various portions of the 5-HT molecule for accumulation of the amine by the myenteric plexus, the accumulation of tritiated 5-HT was measured in the presence of analogs of 5-HT. Concentration-effect curves were obtained for each analog. The ID50 was determined for each inhibitor as well as its relative affinity for the myenteric plexus. The relative affinity (Burgen and Iversen, 1965) was estimated as the reciprocal of the ID50, normalized to give a value of 100 for 5-HT itself. Results of experiments involving the side chain and the alkyl amino group of 5-HT are shown in figure 2. Note that removal of the side chain appeared to remove activity from the compound since 5-hydroxyindole, $10^{-4}$ M, failed to inhibit the accumulation of 5-HT. Methylation of the terminal amino group also greatly decreased activity. The inhibition of 5-HT accumulation by 5-hydroxy-N-methyltryptamine (5-HNMT) appeared to be the result of competitive inhibition and 5-HNMT did not change the measured $V_{max}$ for 5-HT accumulation (fig. 3). However, with increased methylation, the mechanism of inhibition appeared to change and become more

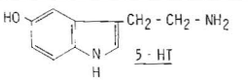
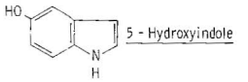
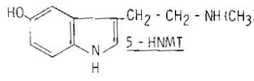
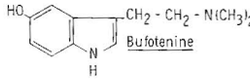
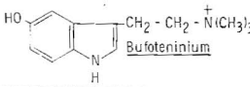
| Compound | $ID_{50}$ | Relative Affinity |
|---|---|---|
|  5 - HT | $7.0 \times 10^{-7}$M ($6.5 - 8.5 \times 10^{-7}$M) | 100 |
|  5 - Hydroxyindole | No inhibition $10^{-4}$M | ~0 |
|  5 - HNMT | $9.1 \times 10^{-5}$M ($7.9 - 13.0 \times 10^{-5}$M) | 0.77 |
|  Bufotenine | $6.5 \times 10^{-5}$M ($4.4 - 9.8 \times 10^{-5}$M) | 1.08 |
|  Bufoteninium | $1.5 \times 10^{-4}$M ($1.0 - 3.6 \times 10^{-4}$M) | 0.46 |

FIG. 2. The effect of related amines on the uptake of labeled 5-HT. Tissues were exposed to the inhibitor for 30 minutes prior to the addition of $^3$H-5-HT. ID50 values were derived from concentration-effect curves for each inhibitor (five to six concentrations of each inhibitor and four to six tissues at each concentration). Numbers in parentheses represent 95% confidence limits of the ID50. The relative affinity refers to the reciprocal of the ID50 normalized to a value of 100 for 5-HT itself.

complex. In the presence of the doubly methylated bufotenine, or the quarternary bufoteninium, the velocity of 5-HT accumulation increased as a sigmoidal rather than a hyperbolic function of the 5-HT concentration (fig. 3). Thus, the mechanism of inhibition by these compounds may not be simply competitive or noncompetitive and may have an allosteric component.

**Effects of substitutions on the indole ring.** Similar experiments involving hydroxylation of the indole ring were done and are summarized in figure 4. Like N-methylation, removal of the hydroxyl group greatly reduces the affinity of the compound for the myenteric plexus and tryptamine is much less effective than 5-HT. Activity is not restored by replacing the hydroxyl group in position 6 and the activity of 6-hydroxytryptamine (6-HT) is the same as that of tryptamine. 5,6-Dihydroxytryptamine (5,6-DHT) was considerably more active than 6-HT; the affinity appeared to be more than 3 times as great. However, the activity of 5,6-DHT was also much less than that of 5-HT itself. This might be taken to indicate that the additional hydroxyl group on the indole ring acts to reduce the activity of the compound, perhaps through steric hindrance. It is also possible that the difference in activity between 5,6-DHT and 5-HT is more apparent than real since 5,6-DHT is much less stable than 5-HT. Another compound which was tested was lysergic acid diethylamide (LSD). As might have been expected from the preceding discussion, LSD, lacking a hydroxyl group on position 5 of its indole ring as well as an aliphatic side chain with a primary amino group, was a very poor inhibitor of 5-HT accumulation. No inhibition was noted up to a concentration of $10^{-5}$ M. Like 5-HNMT, tryptamine and 6-HT appeared to be competitive inhibitors of the accumulation of 5-HT and did not change $V_{max}$.

**Localization of 6-HT taken up by the myenteric plexus.** 6-HT forms a fluorophore upon condensation with formaldehyde vapor that has a higher fluorescent yield than does 5-HT and is easier to detect histochemically (Jonsson *et al.*, 1969). Guinea pigs were subjected to chemical sympathectomy with 6-hydroxydopamine (two injections, 100 mg/kg each, given 24 hours apart) in order to eliminate uptake into adrenergic axons (Thoenen and Tranzer, 1968). 6-Hydroxydopamine treatment eliminated over 90% of fluorescent adrenergic axons. Following incubation for 30 minutes in Krebs' solution containing 6-HT, $10^{-5}$ M, the isolated longitudinal muscle and myenteric plexus were prepared for the histochemical demonstration of monoamines. This treatment with 6-hydroxydopamine has been found not to affect the uptake of 5-HT by the myenteric plexus (Takiff and Gershon, 1970). As can be seen in figure 5, 6-HT was taken up by the myenteric plexus but not by the intestinal smooth muscle. The fluorescence of 6-HT could easily be distinguished from that of residual NE in the plexus, in the small number of areas where NE remained, by the difference in the color of the fluorescence, the diffuse nonvaricose appearance of the fluorescence and the difference in the rate of fading of the respective fluorophores upon exposure to ultraviolet light. Thus, the use of the analog 6-HT reveals that this competitive inhibitor of the uptake of 5-HT actually enters the myenteric plexus. The pattern of fluorescence is widespread throughout the ganglionic regions and is very similar to the pattern previously described for 5-HT (Robinson and Gershon, 1971). It is strikingly dissimi-

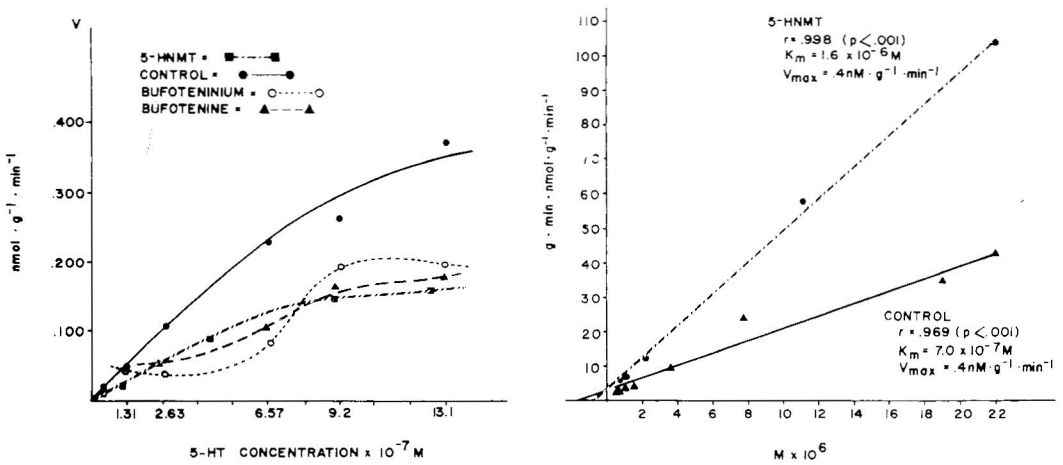



FIG. 3. The effect of analogs on the kinetics of 5-HT uptake. The graph at the left shows the velocity of $^3$H-5-HT uptake plotted as a function of the 5-HT concentration in the absence (control) and presence of the analogs, 5-HNMT, bufotenine and bufoteninium. Each point in the control curve is the mean of 12 to 18 tissues; in the curves for the analogs each point is the mean of eight tissues. Standard errors determined for concentrations of 5-HT below $9 \times 10^{-7}$ M are all $< \pm 0.008$ (smaller than the symbols on the graph). Other standard errors are all $< \pm 0.015$. On the right, the kinetic data are shown for the uptake of 5-HT. The reciprocal of the velocity of $^3$H-5-HT is plotted as a function of the reciprocal of the 5-HT concentration. The coefficient of correlation($r$), P and the $K_m$ and $V_{max}$ are shown for 5-HT uptake in the absence (Control) and the presence of 5-HNMT.

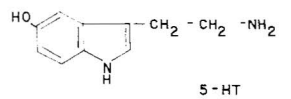
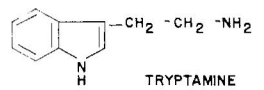
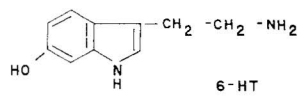
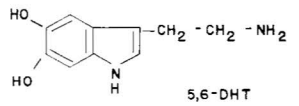
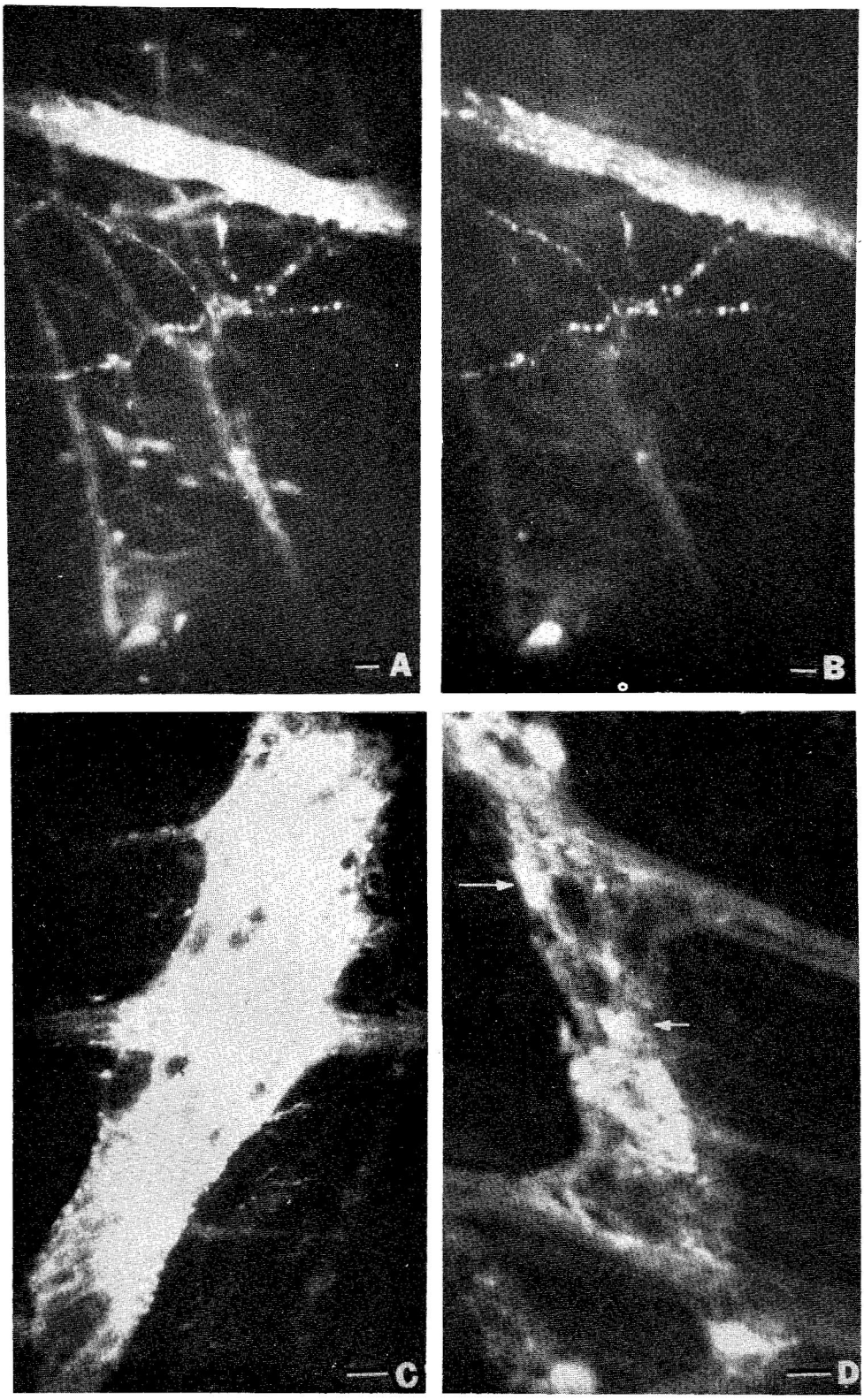
| COMPOUND | $ID_{50}$ | RELATIVE AFFINITY |
|---|---|---|
|  5-HT | $7 \times 10^{-7}$ M ($6.5-8.5 \times 10^{-7}$M) | 100 |
|  TRYPTAMINE | $4.6 \times 10^{-5}$ M ($2.6-6.1 \times 10^{-5}$M) | 1.5 |
|  6-HT | $4.6 \times 10^{-5}$ M ($4.2-5.1 \times 10^{-5}$M) | 1.5 |
|  5,6-DHT | $1.4 \times 10^{-5}$ M ($0.86-2.5 \times 10^{-5}$M) | 4.6 |

FIG. 4. The effect of some related amines on the uptake of 5-HT. The effect of ring substitution is shown. Experiments were performed and the data are presented in the same way as in figure 2. Numbers in parentheses represent 95% confidence limits of the ID50.

lar to the pattern of fluorescence of adrenergic nerves in control preparations of the plexus. In a few regions, occasional perikarya showed 6-HT fluorescence.

**Effect of drugs on the accumulation of amines by the myenteric plexus.** Three tricyclic antidepressants and two amphetamines were examined quantitatively for their ability to inhibit the accumulation of $^3$H-5-HT or $^3$H-NE by elements of the myenteric plexus of the


A
B
C
D

guinea-pig small intestine. Uptake of the tritiated amine was measured as described above and log concentration-effect curves were obtained for the inhibition of amine accumulation for each of the tricyclic antidepressants and the amphetamines. ID50 values and their 95% confidence limits were obtained from the curves. The drugs tested included desmethylimipramine, imipramine, chlorimipramine, methamphetamine and *p*-chloromethamphetamine. Log concentration-effect curves for the tricyclic antidepressants against 5-HT accumulation were parallel to each other for each amine as were the log concentration-effect curves for the amphetamines. The curves for the tricyclic antidepressants, however, were not parallel to those of the amphetamines. ID50 values and relative affinity are shown for $^3$H-5-HT and $^3$H-NE accumulation in table 2. Relative affinity was estimated from the reciprocal of the ID50 (Burgen and Iversen, 1965) normalized to a value for 5-HT or NE as $1/K_m = 100$. Note that all of the drugs were better inhibitors of the accumulation of NE than of 5-HT. The tricyclic antidepressants were good inhibitors of NE accumulation but were relatively weak in their action against 5-HT. The amphetamines were far better inhibitors of the accumulation of 5-HT than were the tricyclic antidepressants while the reverse was true for NE (with the exception of chlorimipramine, a drug which was much less potent against the accumulation of NE than others of its class). The relative effectiveness of the drugs in inhibiting the accumulation of 5-HT as compared with their inhibition of NE accumulation was determined by taking the ratio of the ID50 values for each drug tested. These ratios are listed in table 2. Note in the table that the order of effectiveness of the individual tricyclic compounds against accumulation of 5-HT as compared with their inhibition of NE accumulation is considerably different. Desmethylimipramine is by far the most effective tricyclic antidepressant against the accumulation of NE while chlorimipramine is the most effective tricyclic against accumulation of 5-HT. Thus, the drugs do differentiate between the two amines. However, even the most potent compound against the accumulation of 5-HT, chlorimipramine, could not be used in this system to inhibit the accumulation of 5-HT selectively without at the same time interfering with the accumulation of NE. On the other hand, it probably would be possible to use desmethylimipramine to inhibit accumulation of NE without significantly affecting accumulation of 5-HT.

The mechanism of inhibition of amine accumulation by the drugs was also analyzed. To do this, chlorimipramine and methamphetamine were selected as representative drugs of each class and accumulation of $^3$H-5-HT or $^3$H-NE was measured as a function of the respective amine concentration in the presence of a given concentration of inhibitor. Apparent values for $K_m$ and $V_{max}$ were determined and compared with the values obtained in control experiments done in the absence of inhibitor. The effect of the inhibitors on the kinetics of 5-HT uptake is shown in figure 6 and the results of these experiments are summarized in table 3. For 5-HT, apparent values for both $K_m$ and $V_{max}$ changed significantly (95% confidence limits do not overlap). Therefore, neither inhibitor can be classified as being purely competitive or noncompetitive. For NE on the other hand, $V_{max}$ was unchanged in the presence of chlorimipramine, indicating a competitive mechanism of inhibition. Methamphetamine did change the value of $V_{max}$ for accumulation of NE, but the small change in apparent $K_m$ was not significant and within the limits of experimental error. Thus, inhibition of accumulation in this case was probably largely noncompetitive. The results obtained with the prototypic compounds are likely to characterize the tricyclic antidepressants and amphetamine groups for 5-HT since the log concentration-effect curves for the inhibition of amine accumulation were parallel within each group.

FIG. 5. Histofluorescence of whole mount preparations of myenteric plexus from a guinea pig treated with 6-hydroxydopamine (two 100 mg/kg doses, 24 hours apart, preparations made 24 hours after the second injection) and incubated with 6-HT. The markers = 10 $\mu$m. A. Diffuse fluorescence in the myenteric plexus as well as a few residual fluorescing varicose axons. B. The same field as in A photographed 1 minute later showing that the diffuse fluorescence present in A has faded. The residual adrenergic axons recognizable by thier varicose appearance have not faded. This helps confirm the fact that the diffuse fluorescence is due to the fluorophore formed from the indoleamine, 6-HT. C. Another animal treated identically as described, showing a ganglionic region of the myenteric plexus. The diffuse fluorescence of 6-HT is widespread. D. A ganglionic area from another animal treated as described. Occasional neuronal perikarya (arrows) have picked up the 6-HT and are fluorescent. Such cells are never seen in control tissues not treated with 6-HT.

TABLE 2

*Effect of drugs on $^3$H-amine accumulation*

| | Tricyclic Antidepressants | | | Amphetamines | |
|---|---|---|---|---|---|
| | Imipramine | Desmethylimipramine | Chlorimipramine | Methamphetamine | *p*-Chloromethamphetamine |
| $^3$H-5-HT accumulation | | | | | |
| ID50 (M)[a] | $1.1(0.7 - 1.7) \times 10^{-4}$ | $7.1(5.0 - 10.0) \times 10^{-5}$ | $4.0(2.36 - 6.7) \times 10^{-5}$ | $9.3(2.3 - 3.7) \times 10^{-7}$ | $2.0(0.6 - 6.3) \times 10^{-7}$ |
| Relative affinity | 0.7 | 1.1 | 1.9 | 84.9 | 395 |
| $^3$H-NE accumulation | | | | | |
| ID50 (M)[b] | $6.4(3.2 - 12) \times 10^{-8}$ | $1.7(0.8 - 3.8) \times 10^{-8}$ | $1.3(0.7 - 2.3) \times 10^{-6}$ | $1.8(0.8 - 4.1) \times 10^{-7}$ | $1.0(0.6 - 1.7) \times 10^{-7}$ |
| Relative affinity | 375 | 1411 | 18 | 133 | 240 |
| Ratio ID50 5-HT/ID50 NE | 1719[c] | 4176[c] | 31[d] | 5.2[e] | 2.0[e] |

All ID50 values are reported as means with the calculated 95% confidence limits enclosed in brackets.

[a] The ID50 values for inhibition of $^3$H-5-HT accumulation for all drugs were distinguishable at the 95% confidence level.

[b] The ID50 values for inhibition of $^3$H-NE accumulation for all drugs except imipramine and *p*-chloromethamphetamine were distinguishable at the 95% confidence level.

[c] P(5-HT ID50 *vs.* NE ID50) <.001. [d] P(5-HT ID50 *vs.* NE ID50) <.01. [e] P(5-HT ID50 *vs.* NE ID50) <.05.

Similar results to those described for chlorimipramine were obtained for the inhibition of accumulation of NE by desmethylimipramine. In this case, a value for $K_i$ was obtained by exposing the tissue to different concentrations of desmethylimipramine at two concentrations of $^3$H-NE and measuring the velocity of accumulation of $^3$H-NE. The reciprocal of the velocity of accumulation of NE was then plotted against the desmethylimipramine concentration for each of the two concentrations of NE (method of Dixon, 1953). $K_i$, determined from the point of intersection of the lines corresponding to the two concentrations of NE, was $1.7 \times 10^{-8}$ M. This value is the same as that of the ID50 determined independently for desmethylimipramine. Others have also reported that tricyclic antidepressants like desmethylimipramine competitively inhibit the uptake of NE in peripheral tissues (Iversen and Langer, 1969).

**Release of $^3$H-5-HT by $K^+$ or drugs.** The previously described experiments have measured the net accumulation of $^3$H-5-HT in a stably bound compartment of the myenteric plexus during a 20-minute experimental period. The addition of drugs or a change in the ionic composition of the medium might conceivably decrease this net accumulation by inhibiting uptake of $^3$H-5-HT or accelerating its release. The component of release was evaluated by incubating tissues with $^3$H-5-HT ($9 \times 10^{-7}$ M) for 20 minutes as in previous experiments and then transferring the tissues to fresh vials containing a large volume (10 ml) of experimental or control solutions for a further 20 minutes of incubation. Release of the newly taken up $^3$H-5-HT, accumulated during the previous 20 minutes, in the presence of $K^+$, *p*-chloromethamphetamine or 5,6-dihydroxytryptamine (experimental solutions) was compared with the spontaneous release of $^3$H-5-HT occurring in normal Krebs' (control) solution. These drugs and $K^+$ were selected as prototypes of drugs or ions that might be expected to cause release of $^3$H-5-HT. Concentrations were chosen to be greater than the ID50 values determined for the same substances against accumulation of $^3$H-5-HT. These results are summarized in table 4. Note that none of the experimental solutions caused an appreciably greater release of $^3$H-5-HT over a 20-minute period of exposure than the spontaneous release occurring in normal Krebs' solution.

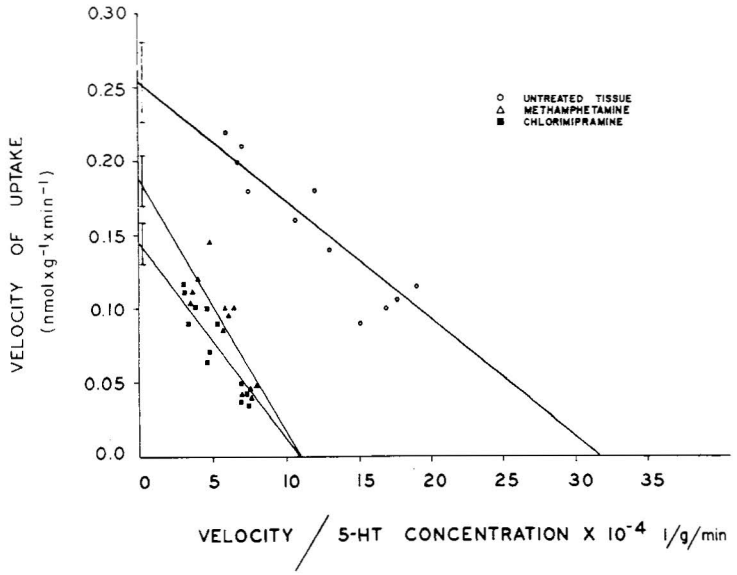



FIG. 6. The velocity of 5-HT uptake is plotted as a function of the relative rate ($V_0/S$). The intersection of the plotted lines on the ordinate represents $V_{max}$ and the slope of the lines, $K_m$. Brackets indicate 95% confidence limits of $V_{max}$.

TABLE 3

*Kinetics of uptake*

Values are reported as means ±95% confidence limit.

| Drug | Inhibitor Concentration | $K_m$ | $V_{max}$ (nmol × $g^{-1}$ × $min^{-1}$ |
|---|---|---|---|
| | (*M*) | (*M*) | |
| $^3$H-5-Hydroxytryptamine | | | |
| None | | $7.9 \pm 4.4 \times 10^{-7}$ | $0.25 \pm 0.03$ |
| Methamphetamine | $5 \times 10^{-6}$ | $17 \pm 8 \times 10^{-7}$ | $0.19 \pm 0.01$ |
| Chlorimipramine | $10^{-4}$ | $13 \pm 9 \times 10^{-7}$ | $0.14 \pm 0.01$ |
| $^3$H-Norepinephrine | | | |
| None | | $2.4 \pm 1.7 \times 10^{-7}$ | $0.15 \pm 0.01$ |
| Methamphetamine | $5 \times 10^{-6}$ | $5.4 \pm 7.0 \times 10^{-7}$ | $0.04 \pm 0.01$ |
| Chlorimipramine | $10^{-6}$ | $13 \pm 2 \times 10^{-7}$ | $0.15 \pm 0.01$ |

## Discussion

Accumulation of $^3$H-5-HT in the myenteric plexus has been studied in these experiments. The experimental design provided a measure of the amount of $^3$H-5-HT in a tissue compartment resistant to washout in physiological solution. Previous radioautographic experiments (Gershon and Altman, 1971) have shown this compartment to be axons of the myenteric plexus. Drugs or $K^+$ conceivably could decrease the amount of $^3$H-5-HT accumulation in these axons either by decreasing the uptake of the amine or by accelerating its release. However, tritiated amine taken up by the axons would mix with the relatively much larger pool of endogenous transmitter. Therefore, before equilibrium is reached, and while the rate of tritium accumulation is linear, the amount of $^3$H-5-HT in the axonal compartment will predominantly reflect influx rather than efflux. The amine taken up will be mainly $^3$H-5-HT while released amine will be diluted by endogenous transmitter. In these experiments the rate of accumulation of $^3$H-5-HT was linear for at least 30 minutes even in the presence of experimental solutions. Thus, the measurements of accumulation of $^3$H-5-HT are probably more sensitive

TABLE 4

*Effect of $K^+$ or drugs on release of $^3$H-5-HT from the guinea pig myenteric plexus*

Tissues were incubated with $^3$H-5-HT ($9 \times 10^{-7}$ M) for 20 min as in the previous experiments on serotonin uptake. They were then transferred to different vials for a further 20-minute incubation at 37°C in control or experimental solutions (10 ml/strip). Following this incubation, radioactivity was extracted from tissues and analyzed. Extracellular space (0.342 ± 12 ml/g) estimated from the $^{14}$C-sorbitol space (Casteels, 1969) and the $^3$H-serotonin content of this space was subtracted from the amount of $^3$H-5-HT found in the washing solutions to correct for washout of free extracellular amine. *% remaining* = $^3$H-serotonin in tissue extract ÷ [$^3$H-5-HT in tissue extract + ($^3$H-5-HT in washing solution − $^3$H-5-HT in extracellular space)] × 100. *% released* = ($^3$H-5-HT in washing solution − $^3$H-5-HT in extracellular space) ÷ [$^3$H-5-HT in tissue extract + ($^3$H-5-HT in washing solution − $^3$H-5-HT in extracellular space)] × 100. Values are means ± S.E. of eight tissues. None of the experimental values differs significantly from control.

| Treatment | $^3$H-5-HT Remaining | | Ratio: (% Released/ % Remaining) |
|---|---|---|---|
| | dpm/g × $10^{-7}$ | % initial uptake | |
| Control | 1.84 ± 0.48 | 35.18 ± 1.01 | 1.85 ± 0.08 |
| KCl (50 mM) | 2.19 ± 0.33 | 36.00 ± 3.97 | 1.73 ± 0.32 |
| *p*-Chloromethamphetamine (1 μM) | 1.62 ± 0.50 | 28.35 ± 4.83 | 2.94 ± 0.85 |
| 5,6-Dihydroxytryptamine (50 μM) | 1.64 ± 0.59 | 34.60 ± 1.89 | 1.86 ± 0.16 |

to interference with uptake rather than release. However, if newly taken up amine were preferentially released by ions or drugs, then release might still appreciably alter net accumulation of $^3$H-5-HT. Release by $K^+$ or prototypic drugs was not in fact greater than the spontaneous release occurring under the conditions prevailing in the current experiments. This failure to detect release is very likely a function of the prolonged exposure of the tissue to the experimental solutions (30 minutes' equilibration, 20 minutes in the presence of $^3$H-5-HT). Catecholamine release by $K^+$ from the adrenal medulla, for example, is a transient phenomenon (Baker and Rink, 1975). It reaches a maximum in 1 minute and then declines with a half-time of about 1.2 minutes despite the continued presence of releasable catecholamine in the gland. Since the depolarization produced by $K^+$ in the adrenal cells is stable, Baker and Rink (1975) postulated that $K^+$ depolarization first activates and then inactivates a potential-sensitive Ca permeability channel. A similar effect of $K^+$ and drugs could explain the current data on release of $^3$H-5-HT from the myenteric plexus. Release by drugs does not appear to have significantly altered the net accumulation of $^3$H-5-HT in the current experiments. Therefore, observed effects of drugs or $K^+$ on $^3$H-5-HT accumulation are probably due to an action on uptake of the amine rather than its release.

The features of the uptake process for 5-HT by elements of the mammalian myenteric plexus have now been characterized in some detail. Considerable evidence supports the view that the uptake is a carrier-mediated process. Prior observations about the process that support this concept are the saturable nature of the process, its high $Q_{10}$, its antagonism by inhibitors of glycolysis, its inhibition by ouabain and its sodium dependence (Gershon and Altman, 1971). Further support for the carrier-mediated nature of 5-HT accumulation has been provided by the present study. There is a considerable specific ion dependence in addition to the previously demonstrated requirement for sodium. Thus, 5-HT accumulation was inhibited by elevating the potassium concentration and also required an optimum calcium concentration, being decreased either in the absence of calcium or in the presence of an excess of that cation. Uptake of NE by synaptosomes has also been reported to have a requirement for calcium but the effect of excess calcium on this system has not been studied (White, 1975). On the other hand, accumulation of 5-HT seemed relatively independent of the concentration of chloride. Additional supportive evidence for the view that uptake of 5-HT is carrier-mediated was provided by the observation that analogs of 5-HT competitively inhibited the accumulation of the amine. Moreover, the accumulation of 5-HT could also be inhibited by drugs such as amphetamines and tricyclic antidepressants. It is still possible that the uptake of 5-HT is due not to the action of a carrier molecule located in

the plasma membrane of axons of the myenteric plexus but to binding of 5-HT by a high molecular weight carrier protein inside the cytoplasm (or within vesicles) of the axons. The uptake could then result from a process of diffusion across the plasma membrane followed by osmotic inactivation of the intracellular amine by binding to the macromolecule and not to a membrane transport protein. Available data are consistent with either possibility and it is not possible to choose with certainty between them at this time. However, in either case, it does seem clear that the myenteric plexus has evolved a rather unique high affinity uptake system for 5-HT ($K_m = 7 \times 10^{-7}$ M), one that differs in several respects from the related uptake process for NE and one which has not been reported anywhere else in the peripheral nervous system.

The structural requirements of the 5-HT molecule for accumulation in the myenteric plexus appear to be quite narrow. In contrast to the uptake of NE by adrenergic axons (Iversen, 1967), small changes in the structure of the 5-HT molecule seem to greatly decrease the affinity of the molecule for the myenteric plexus as judged by the ability of analogs to inhibit accumulation of $^3$H-5-HT. None of the analogs tested in this study even approached the affinity of 5-HT itself. Thus, 5-hydroxyindole had no ability to inhibit uptake of $^3$H-5-HT at all and 5-HNMT, tryptamine and 6-HT had only 0.8 to 1.5% of the affinity of 5-HT. The affinity of 5,6-DHT was greater than that of tryptamine or 6-HT but was less than that of 5-HT. Therefore, these data suggest that an alkyl side chain with a primary amino group, as well as an indole nucleus hydroxylated at position 5, are required for accumulation by the myenteric plexus. Accumulation of 5-HT is thus not inhibited by norepinephrine or indole compounds such as LSD which lack the appropriate structural requirements.

The structure-activity requirements for the accumulation of 5-HT by the neurons of the myenteric plexus are similar to those for uptake of 5-HT by neurons of the central nervous system (CNS). In the central nervous system, as in the myenteric plexus, affinity for the uptake site is reduced by substitution or masking of the terminal amino group or the ring hydroxyl group of 5-HT (Horn, 1973). Affinity is also reduced by placement of the hydroxyl in other than position 5 on the indole ring. 5,6-Dihydroxytryptamine has been reported to be more active in competing with 5-HT for uptake into brain neurons than other hydroxylated tryptamines but reports differ as to whether the affinity of 5,6-dihydroxytryptamine for the uptake site of central nervous system neurons is less than that of 5-HT (Horn *et al.*, 1973) or equal to that of 5-HT (Heikkila and Cohen, 1973).

The rigid structural requirements of the accumulation process probably account for the ability of the elements of the plexus to survive the administration of 6-hydroxydopamine, which can perform a chemical sympathectomy, abolishing uptake of NE by the myenteric plexus, yet not affect the uptake of 5-HT (Takiff and Gershon, 1970). In this respect again, the elements of the myenteric plexus resemble serotonergic neurons of the central nervous system. The high affinity uptake of 5-HT by these CNS neurons is also resistant to inhibition by NE or destruction by 6-hydroxydopamine (Shaskan and Synder, 1970; Kuhar *et al.*, 1972, 1974; Ungerstedt, 1973). It would seem that neurons with an affinity for 5-HT are generally more fastidious in their taste for exogenous amines than are adrenergic neurons.

It is probably also possible to conclude at this time that adrenergic neurons are not responsible for the high affinity specific accumulation of 5-HT in the myenteric plexus. The previously reported differences in susceptibility to 6-hydroxydopamine and the failure of NE to antagonize the uptake of 5-HT certainly support this conclusion. The observations made in this study on the effect of the tricyclic antidepressants and the amphetamines on the accumulation of the two amines in the myenteric plexus are also inconsistent with the hypothesis that the same uptake process could be responsible for the accumulation of both 5-HT and NE. Thus, these drugs differ greatly in the order of potency of their action against the uptake of the two amines. Desmethylimipramine is most effective against the accumulation of NE, while chlorimipramine, the most effective tricyclic antidepressant against the accumulation of 5-HT, is the least effective of these compounds against the accumulation of NE. These observations on the myenteric plexus are qualitatively similar to observations on the effect of these compounds on the uptake of

5-HT and NE by neurons of the CNS (Ross and Renyi, 1967, 1975 a,b; Fuxe and Ungerstedt, 1968; Carlsson, 1970; Meek *et al.*, 1971; Harris and Baldessarini, 1973; Raiteri *et al.*, 1974). All compounds were more effective inhibitors of the accumulation of NE than of 5-HT, a phenomenon also noted for uptake of the amines by slices of midbrain-hypothalamus (Ross and Renyi, 1975b). If the same uptake process were responsible for the uptake of the two amines, then the drugs would have been expected to affect the accumulation of each amine similarly. Their order of potency as uptake antagonists and the mechanisms of their action should not differ. Since this was not the case, the uptake mechanism for NE must not be the same as that for 5-HT.

The significance of the uptake mechanism for 5-HT in the myenteric plexus remains to be determined. Radioautographic studies (Gershon and Ross, 1966) indicate that no other area outside the central nervous system possesses neurons with similar properties. One plausible explanation for the existence of such a unique system in the myenteric plexus is that the serotonergic neurons known to be present in the gut of the lamprey have been retained in mammals. If so, the myenteric plexus should be of considerable interest not only in its own right, but also as a source of accessible serotonergic axons. Confirmation of the existence of serotonergic axons in the myenteric plexus will require the demonstration of additional specific stigmata of these structures, such as the presence within them of tryptophan hydroxylase.

**Acknowledgment.** The authors wish to thank Dr. F. E. Bloom for critical review of the manuscript.

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