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# KINETICS OF SEROTONIN ACCUMULATION INTO SYNAPTOSOMES OF RAT BRAIN—EFFECTS OF AMPHETAMINE AND CHLOROAMPHETAMINES

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**Abstract**—Synaptosomes from whole brain of rats accumulated serotonin by means of a high affinity process and a low affinity process, distinguishable kinetically. The high affinity process was strongly inhibited by 4-chloroamphetamine. 3-Chloroamphetamine, 2-chloroamphetamine and amphetamine were progressively weaker inhibitors of that process. 4-Chloroamphetamine was a less potent inhibitor of the low affinity process (about equal to amphetamine). When 4-chloroamphetamine was administered to rats and synaptosomes were isolated from whole brain, the high affinity uptake of serotonin was inhibited for as long as 22·5 hr after drug administration. *In vitro*, 4-chloroamphetamine but not chloroimipramine at 5 $\mu$M caused a release of serotonin from synaptosomes preincubated with radioactive serotonin. The ability of 4-chloroamphetamine to inhibit the high affinity uptake of serotonin by synaptosomes and to release serotonin from synaptosomes is probably related to the prolonged lowering of brain serotonin levels in rats treated with 4-chloroamphetamine.

SPECIFIC transport systems for neurotransmitter amines and amino acids have been recognized in brain slices and in isolated pinched-off nerve endings (synaptosomes).[1] A high affinity uptake process ($K_m$ 0·17 $\mu$M) has been reported for serotonin (5-HT) in brain slices.[2] The process is largely responsible for the transport of 5-HT at concentrations below 1 $\mu$M. The high affinity for its substrate (5-HT) prevents the interference by catecholamines. Synaptosomes isolated from 6-hydroxydopamine-treated rats have the same transport activity as those from normal animals.[1] As an extension of the above studies, we have compared the effects of 4-chloroamphetamine and amphetamine on the accumulation of 5-HT into synaptosomes, since 4-chloroamphetamine but not amphetamine lowers brain levels of 5-HT.[3,4]

## METHODS AND MATERIALS

*Animals.* Male albino rats derived from the Wistar strain, weighing about 150 g each, were obtained from a local supplier.

*Chemicals and drugs.* DL-[7-$^{14}$C]-norepinephrine (41 mCi/m-mole) and [2-$^{14}$C]-serotonin binoxalate (16 mCi/m-mole) were obtained from New England Nuclear Company. (±)Amphetamine was obtained from Chemicals Procurement Laboratories and (±)4-chloroamphetamine from Regis Chemical Company. (±)2- and (±)3-Chloroamphetamines were prepared by Dr. B. B. Molloy of our laboratories.

*Tissue preparation.* Rats were killed by decapitation, and whole brains were immediately removed. The tissue was homogenized with 9 vol. of ice-chilled 0·32 M sucrose in a glass homogenizer loosely fitted with a motor-driven Teflon pestle. The

homogenate was filtered through two layers of cheesecloth in order to remove fibrous material. The filtrate was homogenized with an additional 15 strokes. A synaptosome-enriched pellet was obtained after centrifugation at 3000 rev/min for 10 min and at 12,000 rev/min for 20 min. The pellet was suspended in 0·32 M sucrose at approximately 25 mg protein/ml and used as the synaptosomal preparation.

*Uptake and release of biogenic amines.* In a temperature-controlled and water-jacketed reaction flask (30°), synaptosomes at 1 mg protein/ml were continuously stirred in 15 ml of Krebs–bicarbonate buffered medium at pH 7·4. After 5 min of equilibration, either [2-$^{14}$C]-serotonin (0·07–2 $\mu$M) or [7-$^{14}$C]-norepinephrine (0·31–0·92 $\mu$M) was added. Three min afterward, four 1-ml samples were immediately filtered through Millipore filters (0·8 $\mu$ pore size) by suction. After rinsing with ice-chilled Krebs–bicarbonate buffer medium, the samples were transferred to counting vials containing 10 ml counting fluid (Permafluor[Packard]–Triton X-100–toluene with a proportion of 1:8:16). Radioactivity was measured in a Packard liquid scintillation spectrometer. Counting efficiency was monitored by internal and external standards.

When the effects of amphetamine and chloroamphetamine were studied, drugs at various concentrations were included during the 5-min equilibration period.

We also examined the effects of 4-chloroamphetamine and chloroimipramine on the release of $^{14}$C-5-HT from synaptosomes. After an incubation of $^{14}$C-5-HT for 7 min, either 4-chloroamphetamine or chloroimipramine was added. Samples of synaptosomes were removed at time intervals for measurement of $^{14}$C-5-HT as before.

## RESULTS

Synaptosomes were incubated with $^{14}$C-5-HT (0·1 $\mu$M) for intervals varying from 10 sec to 5 min. 5-HT accumulation was linear for at least 3 min (Fig. 1) and remained at steady state levels from 5 to 30 min (result not shown). The incubation time of 3 min was chosen for subsequent experiments.

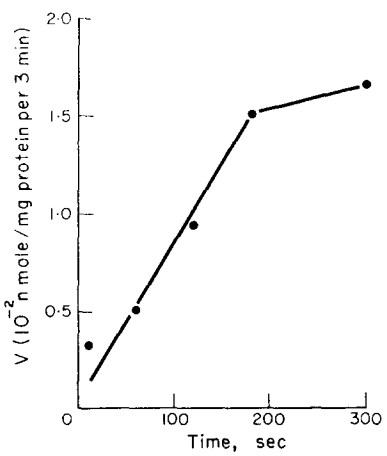


FIG. 1. Time course of $^{14}$C-5-HT accumulation into synaptosomes of rat brain. Reaction mixture consisted of 15 ml Krebs–bicarbonate buffered medium at pH 7·4 and synaptosomes at 1 mg protein/ml. After equilibration for 5 min at 30°, 0·1 $\mu$M $^{14}$C-5-HT was added. Four 1-ml samples were taken at various time intervals and were filtered through Millipore as described in the text.

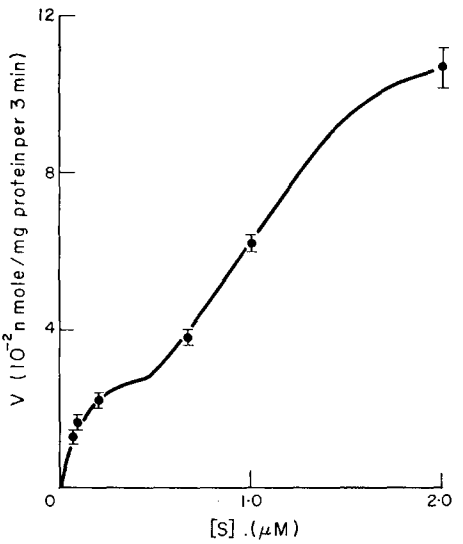


FIG. 2. Rates of accumulation into synaptosomes at various concentrations of $^{14}$C-5-HT. Reaction mixture consisted of 15 ml Krebs–bicarbonate buffered medium, pH 7·4, and synaptosomes at 1 mg protein/ml. After equilibration for 5 min at 30°, $^{14}$C-5-HT (0·07–2 μM) was added. Four 1-ml samples were removed at the end of 3 min and were filtered through Millipore as described in the text.

As pointed out by Shaskan and Snyder,[2] earlier studies of 5-HT uptake by brain slices[5] and synaptosomes[6] had been with concentrations of 5-HT too high to permit recognition of the high affinity uptake process. For the present study, we, therefore, have carefully chosen a range of 5-HT concentration (0·07–2 μM). The accumulation of 5-HT over this concentration range revealed two consecutive saturable curves (Fig. 2). These data were resolved into two straight lines in a Lineweaver–Burk plot (Fig. 3) suggesting at least two components responsible for the accumulation of 5-HT into

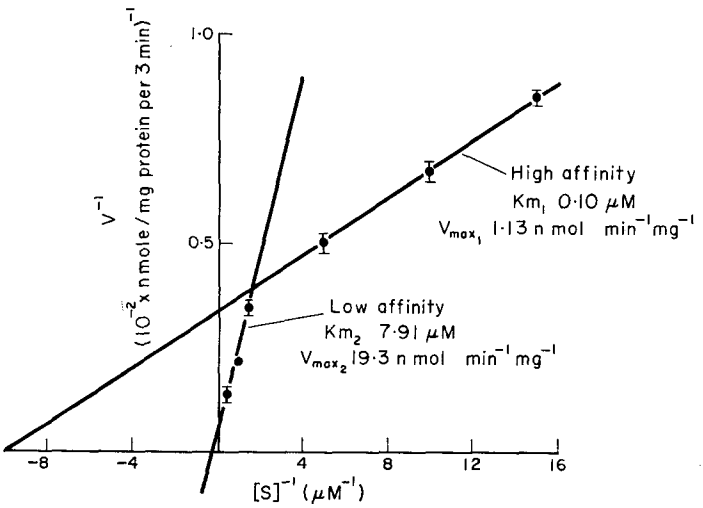


FIG. 3. Lineweaver–Burk plot on data shown in Fig. 2.

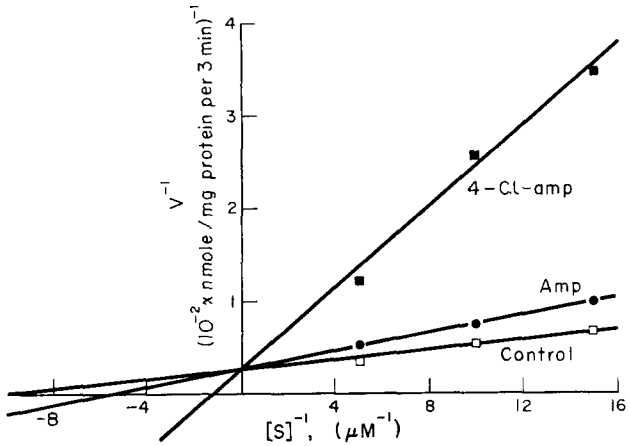


FIG. 4. Competitive inhibition of the high affinity uptake of $^{14}$C-5-HT by 4-chloroamphetamine and amphetamine as illustrated by the Lineweaver–Burk method. Reaction conditions were the same as in Fig. 2 except that 4·9 $\mu$M 4-chloroamphetamine (■) or 5·4 $\mu$M amphetamine (●) was included during temperature equilibration (□ indicates control rates). Concentration range of $^{14}$C-5-HT was 0·07–0·2 $\mu$M.

synaptosomes. One component was operative at low concentrations of 5-HT with $K_{m_1}$ at 0·10 $\mu$M (high affinity uptake) and another component with $K_{m_2}$ at 7·91 $\mu$M (low affinity uptake). The respective values reported by Shaskan and Snyder[2] for brain slices were 0·17 and 8 $\mu$M. Both components of 5-HT uptake into synaptosomes were sensitive to metabolic inhibitors such as potassium cyanide and 2,4-dinitrophenol (unpublished data).

TABLE 1. KINETIC PARAMETERS OF $^{14}$C-5-HT UPTAKE BY RAT BRAIN SYNAPTOSOMES*

| Compounds | Kinetic constants | | | |
|---|---|---|---|---|
| | $K_{m_1}$ ($\mu$M) | $K_{i_1}$ ($\mu$M) | $K_{m_2}$ ($\mu$M) | $K_{i_2}$ ($\mu$M) |
| Serotonin | 0·10 ± 0·01 | | 7·91 ± 0·17 | |
| (±)Amphetamine, 5·4 $\mu$M | 0·18 | 6·99 | 9·1 | 6·64 |
| (±)4-Chloroamphetamine, 4·9 $\mu$M | 0·91 | 0·62 | 14·3 | 2·63 |

* Data were originated from experiments shown in Figs. 3–9.

4-Chloroamphetamine lowers brain 5-HT levels in rats, whereas amphetamine has no effect.[3,4] Carlsson[7] has shown that 4-chloroamphetamine, like chloroimipramine, is a potent inhibitor of 5-HT uptake into brain slices. We thought 4-chloroamphetamine might specifically inhibit the high affinity uptake of 5-HT into serotoninergic neurons.[2] The results of the experiment shown in Fig. 4 and summarized in Table 1 support this idea. 4-Chloroamphetamine (4·8 $\mu$M) greatly increased the $K_m$ for the high affinity uptake process (from 0·10 to 0·91 $\mu$M) whereas amphetamine (5·4 $\mu$M)

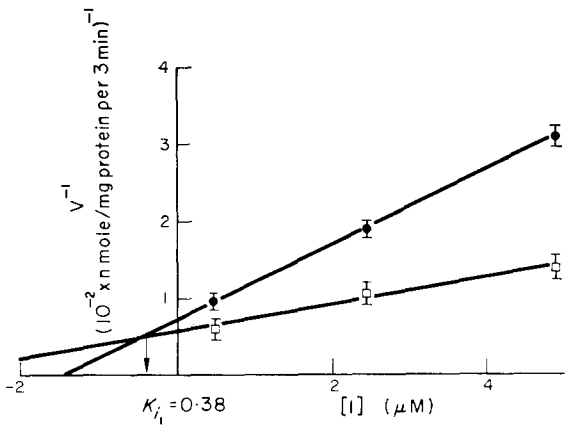


FIG. 5. Competitive inhibition of the high affinity $^{14}$C-5-HT uptake by 4-chloroamphetamine. Determination of $K_i$ by the method of Dixon.[8] Reaction mixture consisted of 15 ml Krebs-bicarbonate buffered medium at pH 7·4 and synaptosomes at 1 mg protein/ml. During the equilibration period, three concentrations of 4-chloroamphetamine (0·48–4·8 $\mu$M) were used and either 0·1 $\mu$M (●) or 0·2 $\mu$M (□) $^{14}$C-5-HT was added to start the uptake process. At the end of 3 min with $^{14}$C-5-HT, four 1-ml samples were removed and filtered as described in the text.

had only a minor effect on that $K_m$ (increased to 0·18 $\mu$M). Separate experiments were designed to obtain $K_i$ values for the two drugs by the method of Dixon.[8] The $K_i$ for 4-chloroamphetamine was 0·38 $\mu$M (Fig. 5) and 0·62 $\mu$M estimated from $K_{m_1}$ and known inhibitor concentration (Table 1) and that for amphetamine was about 7 $\mu$M (Fig. 6). Both drugs were competitive inhibitors, as evidenced from Fig. 4 as well as Figs. 5 and 6.

4-Chloroamphetamine and amphetamine were also competitive inhibitors of the low affinity uptake process for 5-HT (Fig. 7), though 4-chloroamphetamine was again a better inhibitor than amphetamine. Experiments that yielded Dixon plots

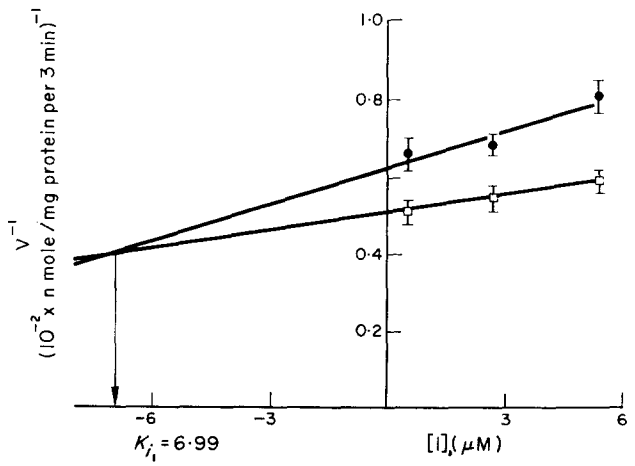


FIG. 6. Competitive inhibition of the high affinity of $^{14}$C-5-HT uptake by amphetamine. Determination of $K_i$ by the method of Dixon.[8] Reaction conditions and symbols were the same as in Fig. 5 except that amphetamine (0·54–5·4 $\mu$M) was used.

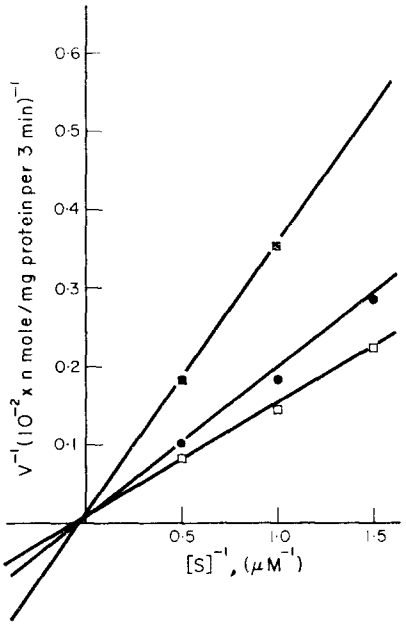


FIG. 7. Competitive inhibition of the low affinity uptake of $^{14}$C-5-HT by 4-chloroamphetamine and amphetamine as illustrated by the Lineweaver–Burk method. Reaction conditions were the same as in Fig. 2 except that 4·9 $\mu$M 4-chloroamphetamine (■) or 5·4 $\mu$M amphetamine (●) was included during temperature equilibration (□ indicated control rates). Concentration range of $^{14}$C-5-HT was 1–2 $\mu$M.

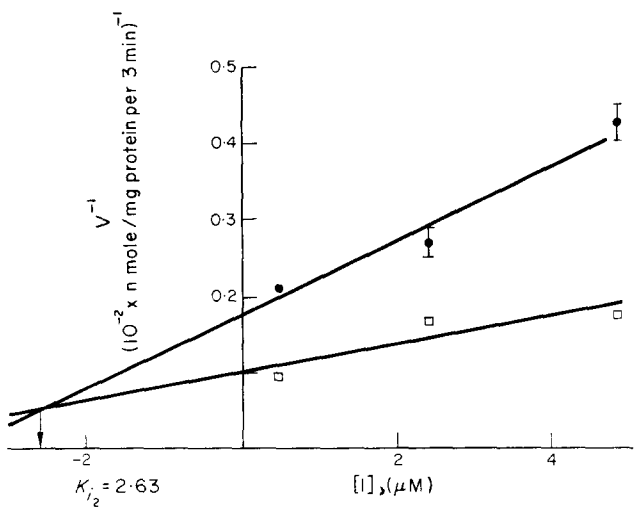


FIG. 8. Competitive inhibition of the low affinity $^{14}$C-5-HT uptake by 4-chloroamphetamine. Determination of $K_i$ by the method of Dixon.[8] Reaction conditions were the same as in Fig. 5 except that concentrations of $^{14}$C-5-HT were 1 $\mu$M (●) and 2 $\mu$M (□).

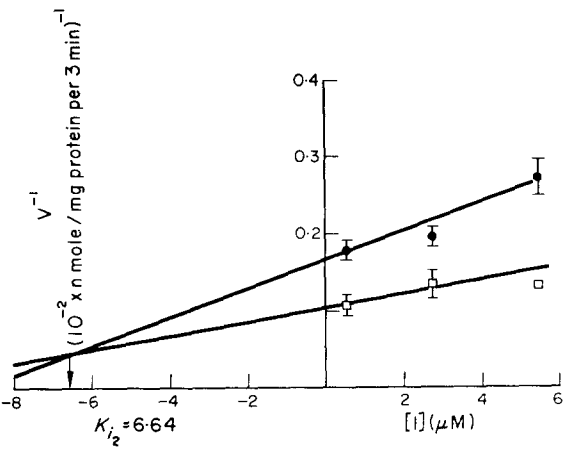


FIG. 9. Competitive inhibition of the low affinity $^{14}$C-5-HT uptake by amphetamine. Determination of $K_i$ by the method of Dixon.[8] Reaction conditions and symbols were the same as in Fig. 8 except that amphetamine (0·54–5·4 $\mu$M) was used.

showed that 4-chloroamphetamine inhibited the low affinity uptake with a $K_i$ of 2·63 $\mu$M (Fig. 8) compared to the $K_i$ of 6·64 for amphetamine (Fig. 9). These values are also summarized in Table 1.

Effects of other chloro-substituted amphetamine were examined and the results are shown in Table 2. 4-Chloroamphetamine was the most potent in blocking the high

TABLE 2. EFFECTS OF AMPHETAMINE AND CHLORO-DERIVATIVES ON $^{14}$C-5-HT UPTAKE PROCESSES OF RAT BRAIN SYNAPTOSOMES*

| | High affinity uptake | | Low affinity uptake | |
|---|---|---|---|---|
| Amphetamine analog added | Rate (nmoles mg protein$^{-1}$ 3 min$^{-1}$) | Control (%) | Rate (nmoles mg protein$^{-1}$ 3 min$^{-1}$) | Control (%) |
| None | 1·91 ± 0·05 | 100 | 6·59 ± 0·10 | 100 |
| Amphetamine | 1·39 ± 0·02 | 72·6 | 4·56 ± 0·27 | 69·2 |
| 2-Cl-Amphetamine | 1·10 ± 0·07 | 57·8 | 4·22 ± 0·17 | 64·1 |
| 3-Cl-Amphetamine | 0·72 ± 0·03 | 37·9 | 4·27 ± 0·15 | 64·9 |
| 4-Cl-Amphetamine | 0·42 ± 0·02 | 21·8 | 3·56 ± 0·07 | 54·1 |

* Reaction conditions were the same as in Fig. 2 except that two specific concentrations of $^{14}$C-5-HT were selected for the high affinity uptake (0·1 $\mu$M) and the low affinity uptake (1 $\mu$M). The concentration of amphetamine was 5·4 $\mu$M and those for the chloro-derivatives were 4·8 $\mu$M.

affinity uptake of 5-HT (22 per cent of control rate). 3-Chloroamphetamine, 2-chloroamphetamine, and amphetamine were less effective in a descending order of potency. On the other hand, all four drugs inhibited the low affinity uptake of 5-HT (1 $\mu$M) to the same degree. Thus, the effects of the four drugs on the high affinity uptake process of serotonin paralleled their effects on the brain serotonin levels *in vivo*.[9,10] The high affinity uptake process was seemingly specific for 5-HT, and it was

likely through this pathway that the brain 5-HT was released under the influence of 4-chloroamphetamine *in vivo*.

We reported earlier that 4-chloroamphetamine was bound to synaptosomal fraction of whole brain homogenate.[11] Perhaps, that tightly bound molecule of 4-chloroamphetamine exerts inhibitory effects on the specific uptake of 5-HT. Therefore, we examined the effects of washing of the synaptosomes isolated from rats treated with 4-chloroamphetamine for 1 and 22·5 hr (Table 3). Synaptosomes isolated from

TABLE 3. EFFECTS OF WASHING ON DRUG LEVELS AND $^{14}$C-5-HT UPTAKE PROCESSES OF SYNAPTOSOMES ISOLATED FROM 4-CHLOROAMPHETAMINE AND AMPHETAMINE-TREATED RATS*

| Synaptosomal preparation | Drug levels (μg/mg) | Uptake processes of 5-HT‖ | |
|---|---|---|---|
| | | High affinity (0·1 μM 5-HT) | Low affinity (1 μM 5-HT) |
| Control, unwashed | None | 1·68 ± 0·03 | 6·28 ± 0·19 |
| One hr after amphetamine administration | | | |
| Unwashed | 0·13 | 1·53 ± 0·05† | 3·58 ± 0·30‡ |
| Washed | 0·08 | 1·71 ± 0·06 | 6·25 ± 0·10 |
| One hr after 4-Cl-amphetamine administration | | | |
| Unwashed | 0·62 | 0·53 ± 0·03‡ | 3·49 ± 0·28‡ |
| Washed | 0·49 | 0·66 ± 0·01‡ | 4·52 ± 0·20‡ |
| Twenty-two and one-half hr after 4-Cl-amphetamine administration | | | |
| Unwashed | 0·12 | 0·62 ± 0·05‡ | 4·26 ± 0·32§ |
| Washed | 0·10 | 0·91 ± 0·04‡ | 6·47 ± 0·34 |

* Synaptosomes were isolated from rats pretreated with equimolar doses of amphetamine (18 mg/kg) for 1 hr and 4-chloroamphetamine (24 mg/kg) for 1 and 22·5 hr. Portions of synaptosomes were washed twice with 10-fold vol. of 0·32 M sucrose by repeated suspension and centrifugation. The high and low affinity uptake processes were examined at 0·1 and 1 μM $^{14}$C-5-HT. Other conditions were the same as described for Fig. 2. Drug levels were measured by the methyl orange method.[9]

† $P > 0·05$.

‡ $P > 0·001$.

§ $P > 0·005$.

‖ nmole mg protein$^{-1}$ 3 min $^{-1}$.

amphetamine-treated rats for 1 hr were also examined for comparison. The amount of amphetamine present in synaptosomes was only about 0·13 μg/mg and 60 per cent of it resisted extensive washing. The rates of 5-HT uptake were, however, restored completely by washing. On the other hand, 1 hr after administration the amount of 4-chloroamphetamine was 0·62 μg/mg and was not removed by washing of the synaptosomes. Although washing might have increased the specific activities for the high and low affinity uptake processes of 5-HT, they were only 40 and 70 per cent of their control values. At the end of 22·5 hr after administration, the amount of 4-chloroamphetamine in the synaptosomes was 0·12 μg/mg and was largely unremovable by washing. The high affinity uptake of 5-HT was yet inhibited by 45 per cent while the low affinity uptake was completely restored to normal rate by washing.

The effects of 4-chloroamphetamine and chloroimipramine on the release of 5-HT from synaptosomes were compared (Fig. 10). After an incubation of synaptosomal

preparation with $^{14}$C-5-HT for 7 min, either 4-chloroamphetamine or chloroimipramine at 5 and 10 $\mu$M was added. The solid lines show the amounts of $^{14}$C-5-HT accumulated or remained during incubation with and without drugs. The dotted lines show the difference between the amounts of $^{14}$C-5-HT in the treated synaptosomes at a specific time and the control amount (19 pmoles/mg) which was the extrapolated level in 7 min of incubation without drug. That difference represented the amount

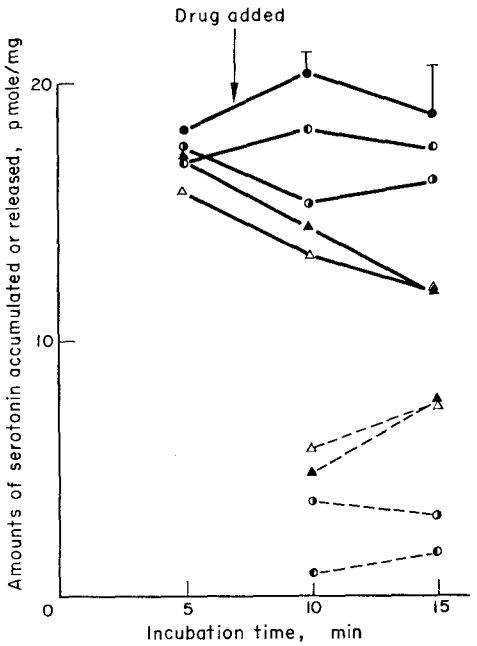


FIG. 10. Effects of 4-chloroamphetamine and chloroimipramine on the release of 5-HT from synaptosomes. Synaptosomes were pre-loaded with $^{14}$C-5-HT (0·1 $\mu$M) as in Fig. 2. The accumulated levels of $^{14}$C-5-HT were monitored at 5, 10 and 15 min after the addition of the radioactive substrate. Either 4-chloroamphetamine or chloroimipramine was added in 7 min (arrow) when about 19 pmoles $^{14}$C-5-HT was normally accumulated. The solid lines represent $^{14}$C-5-HT released from synaptosomes. Symbols represent: control (●); 4-chloroamphetamine at 5 (△) and 10 $\mu$M (▲); and chloroimipramine at 5 (◐) and 10 $\mu$M (◑).

released due to the effects of drugs. Three min after the addition of 5 $\mu$M 4-chloroamphetamine, 4·5 pmoles/mg of 5-HT was released. However, equal molar chloroimipramine did not cause significant amounts of 5-HT released in 3 min but it did induce 5-HT release (2 pmoles/mg) in 8 min after the addition of the drug. Figure 11 shows that the threshold concentration of 4-chloroamphetamine to cause 5-HT release was about 2·5 $\mu$M. Chloroimipramine induced 5-HT release only at higher concentration, 10 $\mu$M.

We also found that norepinephrine was accumulated into synaptosomes with $K_m$ 0·63 $\mu$M (Table 4) which was about 6-fold larger than the $K_m$ for 5-HT via the high affinity process ($K_{m_1}$, 0·10 $\mu$M). The norepinephrine uptake was also competitively inhibited by (±) amphetamine and 4-chloroamphetamine having $K_i$ values of 0·52 and 0·84 $\mu$M respectively (Table 4). Thus these two drugs were equally effective

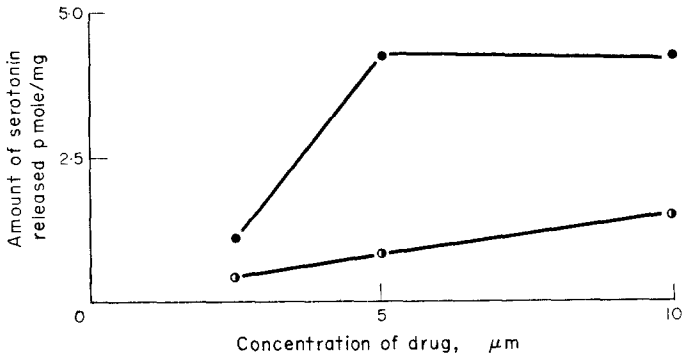


FIG. 11. The amount of 5-HT release induced by various concentrations of 4-chloroamphetamines and chloroimipramine. Experimental conditions were identical to those described for Fig. 10, except that the released amounts of $^{14}$C-5-HT were those at 15 min of incubation or 12 min after the addition of various concentrations of 4-chloroamphetamine (●) or chloroimipramine (◑).

inhibitors of norepinephrine uptake. In other words, the only difference observed between amphetamine and 4-chloroamphetamine was the greater degree of inhibition in 5-HT uptake by 4-chloroamphetamine via the high affinity uptake process.

TABLE 4. KINETIC PARAMETERS OF $^{14}$C-NOREPINEPHRINE UPTAKE BY RAT BRAIN SYNAPTOSOMES*

| Compounds | Kinetic constants | | |
|---|---|---|---|
| | $K_m$ (μM) | $K_i$ (μM) | $V_{max}$ (nmoles/min/mg protein) |
| (±)Norepinephrine | 0·63 ± 0·06 | | 2·95 ± 0·02 |
| (±)Amphetamine 5·4 μM | 7·21 | 0·52 | |
| 4-Chloroamphetamine 4·9 μM | 4·32 | 0·84 | |

* For determination of $K_m$ ($K_m'$), the experimental conditions were the same as in Fig. 2 except that $^{14}$C-norepinephrine (0·3–1·4 μM) was used as substrate. Separate experiments were made to determine $K_i$ by the method of Dixon.[8] In these experiments, two specific concentrations of $^{14}$C-norepinephrine (0·146 and 1·4 μM) and various concentrations of amphetamine (0·54–5·4 μM) and 4-chloroamphetamine (0·48–4·8 μM) were used.

## DISCUSSION

In the present study of 5-HT accumulation into synaptosomes, we have confirmed the dual transport processes described by Shaskan and Snyder[2] who studied brain slices. We are in agreement with their conclusion that the high affinity uptake process is a distinct mechanism specific for 5-HT and that the low affinity uptake of 5-HT may represent the uptake of the amine into catecholaminergic neurons. We have found that the high affinity uptake of 5-HT can be selectively inhibited by 4-chloroamphetamine to a greater degree than the inhibition of the low affinity uptake of 5-HT or the uptake of catecholamines.

We have observed the following pharmacological differences between 4-chloroamphetamine and amphetamine. First, the former drug is more potent in inhibiting

the high affinity uptake of 5-HT. Second, 4-chloroamphetamine tightly binds to synaptosomes for as long as 22·5 hr after administration *in vivo*. Third, the residual amount of 4-chloroamphetamine (0·10 μg/mg) at 22·5 hr after its administration inhibits the specific uptake of 5-HT by 45 per cent. On the other hand, only one-fifth the amount of amphetamine is found in synaptosomes after 1 hr after administration *in vivo*. The inhibition of both 5-HT uptake processes by the residual amphetamine can be removed by washing. These observations may be related to the ability of 4-chloroamphetamine but not amphetamine to reduce levels of 5-HT in whole brain.

Two other observations made in the present study may support the above conclusion. 3-Chloroamphetamine is near 4-chloroamphetamine in potency of inhibition of the high affinity uptake and in reducing brain 5-HT levels in rats[9] and guinea pigs.[10] In addition, 2-chloroamphetamine is closer to amphetamine in its relatively weak inhibition on uptake of 5-HT and in its inability to lower brain 5-HT.[9,10]

The potent inhibition of 5-HT uptake by 4-chloroamphetamine might represent competition between two substrates for the "5-HT pump", i.e. 4-chloroamphetamine itself may be highly concentrated by serotoninergic neurons. We have recently published evidence that 4-chloroamphetamine is accumulated into brain synaptosomes after its administration to rats[11] but that study, of course, did not indicate any specific association with serotoninergic synaptosomes. A study *in vivo* has been reported that chloroimipramine prevents the 5-HT lowering effect of 4-chloromethamphetamine.[12] This has been interpreted as evidence for the inhibition of 4-chloromethamphetamine accumulation into the neurons by chloroimipramine. Perhaps, chloroimipramine would prevent the 5-HT lowering effect of 4-chloroamphetamine in the same manner.

After its accumulation into neurons, 4-chloroamphetamine may exert its influence in the following ways. It might release the endogenous 5-HT as we have observed in the present study. The re-uptake of 5-HT is blocked by the same drug even at much lower concentrations. The released 5-HT diffuses into circulation and a lower brain 5-HT level results. When sufficient internal concentration of 4-chloroamphetamine is reached, it may lower 5-HT levels by an alternate mechanism such as inhibition of tryptophan hydroxylase as proposed by Sanders–Bush and Sulser.[13–15] Consistent with this notion is the finding by those authors that 4-chloroamphetamine is highly specific as an inhibitor of brain tryptophan hydroxylase and does not affect the enzyme in liver; in contrast, *p*-chlorophenylalanine inhibits both brain and liver tryptophan hydroxylases.[16,17]

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