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Displacement of Serotonin from Its Adenosine Triphosphate Complex by Tricyclic Antidepressant Drugs *in Vitro*

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SUMMARY

NOGRADY, THOMAS, NOGRADY, HEATHER I., HRDINA, PAVEL D. & LING, GEORGE M. (1975) Displacement of serotonin from its adenosine triphosphate complex by tricyclic antidepressant drugs *in vitro*. *Mol. Pharmacol.*, 11, 795–802.

Serotonin forms a high molecular weight micellar complex with ATP, which is assumed to be the storage form of the neurotransmitter in nerve ending granules. Since bound serotonin (5-HT) does not show fluorescence, complex formation is accompanied by quenching of its fluorescence. Using a solution of a 5-HT–ATP complex at a 1:2 molar ratio containing 5 mM 5-HT, we observed fluorescence enhancement upon addition of tricyclic antidepressants in proportion to the amount and nature of the drug. The molar ratio of drug to 5-HT necessary to liberate 50% of bound serotonin was used to characterize the relative efficacy of the drugs in competing with 5-HT for ATP. The drugs also form water-insoluble complexes with ATP in a 2:1 molar ratio even in the presence of 5-HT. The solid complexes were characterized by infrared and NMR spectroscopy as well as by elemental analysis. The liberation of 5-HT from its "storage complex" *in vitro* suggests that an intraneuronal mode of action of antidepressant drugs is possible, in addition to their known inhibition of reuptake at the presynaptic neuronal membrane.

INTRODUCTION

It is widely assumed that serotonin (5-hydroxytryptamine) deficiency is associated with clinical depressive syndromes (1). The storage, release, and uptake of this neurotransmitter are believed to be intimately involved in the action of tricyclic antidepressant drugs on the central nervous system. Some of these drugs primarily influence serotoninergic synapses [imipramine (2), chlorimipramine (3), and amitriptyline] while others [desipramine (4)] seem to interact predominantly with the central adrenergic system. However, their mode of action on either serotoninergic or noradrenergic neurons appears to be identical, by blockage of the membrane pump responsible for uptake of neurotransmitter through the presynaptic membrane (5, 6). Inhibition of this process—a major means of inactivating the released neurotransmitter—results in increased availability of neurotransmitter in the synaptic gap for action on the postsynaptic receptor site.

However, antidepressants may have in addition an intraneuronal site of action. Desipramine was shown to prevent uptake

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of exogenous norepinephrine (7) and [^{3}H]tyramine (8) into synaptic storage vesicles. Furthermore, imipramine, chlorimipramine, and amitriptyline, but not desipramine, are capable of releasing 5-HT[1] from brain tissue slices (9). The intraneuronal effect of imipramine-like compounds could therefore be related to their interference with the uptake of neurotransmitters into and/or release from the synaptic storage granules (10, 11) or extragranular pools.

This report provides evidence that tricyclic antidepressant drugs interfere with 5-HT storage by competing for ATP in the formation of 5-HT–ATP micelles. The existence of these high molecular weight aggregates (up to 12,000–14,000 mol wt) was first shown by Pletscher and co-workers (12) in blood platelet storage organelles, as well as in synaptic vesicles. The micelles form spontaneously and are specific for 5-HT, their size being a function of temperature as well as Mg^{++} and Ca^{++} concentrations. Similar storage complex formation for norepinephrine (13, 14), acetylcholine (15) and epinephrine (16) has also been demonstrated. Recently we investigated the structure and binding mechanism of the 5-HT–ATP micelles *in vitro* by NMR methods and proved the involvement of ionic and contact charge-transfer interactions (17). Since the NMR method is not suitable for studying the effects of drugs on these aggregates, we also examined the same phenomenon by fluorescence titration of 5-HT with ATP (18). The 5-HT bound to ATP loses its fluorescence. Therefore, if a third component (e.g., a drug) is capable of displacing 5-HT from its complexed, nonfluorescing "storage form," a fluorescence enhancement will be observed as a result of the presence of increased amounts of free 5-HT. In the present paper we show such a titration of a 5-HT–ATP complex *in vitro* with several antidepressant drugs, resulting in the liberation of 5-HT from its complex.

That the drugs do indeed form complexes with ATP was demonstrated by the isolation of water-insoluble drug-ATP complexes. Most of these are amorphous solids, not easily amenable to precise analysis. However, the complex of imipramine with ATP is crystalline, as described below.

[1] The abbreviation used is: 5-HT, serotonin (5-hydroxytryptamine).

MATERIALS AND METHODS

Chemicals and drugs. Serotonin oxalate and ATP·2Na·4H_2O were purchased from ICN Pharmaceuticals, Inc., and kept at 0° in darkness; fresh solutions were prepared daily. Drugs used were the gracious gifts of Ayerst, McKenna & Harrison, Ltd., Montreal (amitriptyline), Eli Lilly, Inc. (nortriptyline), Ciba-Geigy (Canada), Ltd. (imipramine, desipramine, chlorimipramine, and 10,11-didehydroimipramine, G 31406), and Poulenc Frères, Ltée., Montreal (chlorpromazine). All drugs were used as received, in the form of their hydrochlorides.

Instrumentation. Fluorescence spectra were recorded on a Perkin-Elmer 204 double-grating instrument equipped with 1-mm microcells. The same cell was used throughout the whole investigation. The temperature of the sample compartment was kept at 23° ± 0.5° by blowing in a stream of cold air. Infrared spectra were recorded on a Beckman IR-8 instrument in KBr pellets. NMR spectra were recorded on a Varian T-60 instrument in D_2O at probe temperature with internal standard. Analyses were performed by Dr. C. Daesslé (Montreal); phosphorus determinations were done in our own laboratory, using both inorganic orthophosphate and hydrolyzed ATP as standards. All compounds were digested in 5 ml of 70% perchloric acid for 25–50-mg samples under reflux for 2 hr to avoid formation of pyrophosphate and, consequently, low phosphorus values. Sodium was determined by flame photometry. The pH of solutions was 3.5.

Fluorescence titrations. A 5-HT–ATP complex solution in a molar ratio of 1:2 was prepared containing 10 mM 5-HT oxalate and 20 mM ATP·2Na·4H_2O. Aliquots of this solution were weighed into vials on an analytical balance (to avoid pipette error) and diluted with an equal weight of water (for complex blank) or an appropri-

ate amount of 0.1 M drug solution (the "titrant") plus water. This gave a constant final 5 mM 5-HT concentration while the drug to 5-HT molar ratio was varied between 10:1 and 0.1:1. The solutions were thoroughly mixed and then centrifuged at low speed in the same vial to remove eventual suspended drug-ATP complex, which separated at high drug concentrations. The microcuvette was flushed at least four or five times with the supernatant, using a syringe, and its fluorescence was read five to seven times at 305 nm excitation and 345 nm emission wavelengths, to decrease the inner filter effect of the drug. A 2-min waiting period between readings was necessary to allow for recovery of the 5-HT fluorescence. To correct for the inner filter effect of the drugs, a series of blanks was prepared containing 50 mM 5-HT and the appropriate concentration of drug, varying between 50 and 0.5 mM. A 5 mM 5-HT blank was also run with every series, prepared from the same stock solution as used for the 5-HT–drug blanks. One drug series and all necessary blanks were run the same day.

The corrected percentage emission was obtained by subtracting the 5-HT–drug blank fluorescence from the pure 5-HT fluorescence and adding this difference to the percentage emission of the three-component system. The titration curves are a plot of corrected percentage emission vs. drug to 5-HT ratio. The midpoint between the values for 5-HT and 1:2 5-HT–ATP fluorescence gives the drug to 5-HT ratio at which the drug displaced 50% of bound 5-HT, and is the ED_{50} value used to characterize the drug. Determinations were run in triplicate.

In the series containing Mg^{++} an amount of $MgCl_2 \cdot 6H_2O$ equimolar to ATP was incorporated in all solutions, including blanks.

RESULTS

Fluorescence titrations. Displacement of 5-HT from 5-HT–ATP complex solutions by tricyclic antidepressant drugs was demonstrated by observing the fluorescence enhancement due to liberation of free, noncomplexed 5-HT upon addition of drug to a 1:2 5-HT–ATP solution containing 5 mM 5-HT. Such an equilibrium solution showed approximately 50% quenching of serotonin fluorescence (Fig. 1) (18). The drugs investigated were of the dibenzocycloheptene and dibenzoazepine type with identical mono- or dimethylaminopropyl side chains: amitriptyline, nortriptyline, imipramine, chlorimipramine, desmethylimipramine, and G 31406 (the Δ^{10} unsaturated analogue of imipramine; see ref. 19).

As increasing amounts of drug are added, they compete for ATP, liberating free fluorescent 5-HT and forming the presumably more stable drug-ATP complexes. The course of such titrations is shown in Fig. 2 for four drugs of the imipramine family. Since we were dealing with complicated multiple equilibria, no attempt was made to calculate association constants. We simply determined ED_{50} values for each drug, which represent the drug to 5-HT molar ratio necessary to liberate 50% of bound serotonin. The values are shown in Table 1 for all drugs used. The values for the zero point (i.e., no drug present) and for pure 5-HT vary somewhat, since a new 5-HT–ATP solution was prepared for each run. Because of the method of calculation, ED_{50} values were not influenced by this fact.

In the same table ED_{50} values in the presence of 10 mM $MgCl_2$ are shown. It was conceivable that Mg^{++} ions, having a

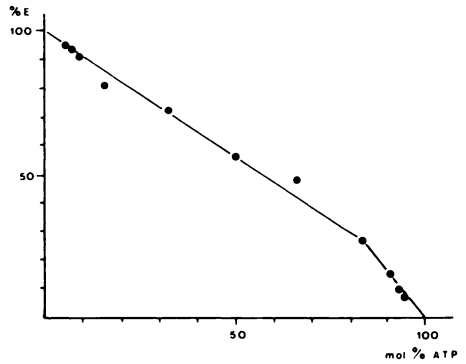

FIG. 1. *Fluorescence quenching of serotonin by ATP*

Percentage emission (%*E*) of 5-HT is plotted against ATP concentration. The 5-HT concentration was 5 mM in a 1-mm microcuvette. Excitation, 305 nm; emission, 345 nm; at 23°.

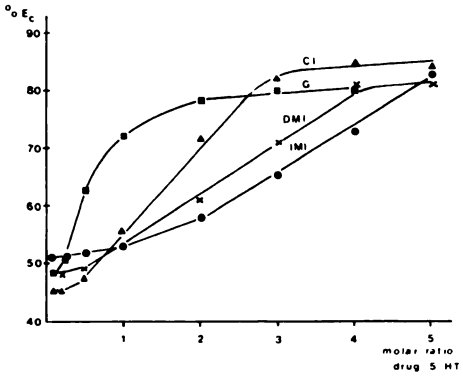

FIG. 2. *Fluorometric titration curves of imipramine derivatives*

Percentage emission corrected for inner filter effects ($\%E_c$) is plotted against drug to 5-HT molar ratio. A 1:2 5-HT–ATP solution containing 5 mM 5-HT was titrated. The fluorescence of this solution is the value at zero drug to 5-HT ratio. Not shown are data from experiments performed with 7.5- and 10-fold drug excesses. IMI, imipramine; DMI, desipramine; CI, chlorimipramine; G, G 31406.

strong affinity for ATP, might influence drug-ATP binding. In most cases the ED_{50} values were very close to those observed in the absence of Mg^{++}, with the exception of amitriptyline, for which a large increase in ED_{50} (i.e., decreased binding to ATP) was observed.

Crystalline antidepressant-ATP complexes. During the preparation of solutions for spectrofluorometry, formation of precipitates was observed. On a preparative scale, imipramine gave the best-defined complexes in the following way. Solid imipramine HCl and ATP·2Na, in a molar ratio varying from 1:1 to 4:1 and weighing a total of 250 mg, were placed in a test tube, and 1–3 ml of water were added dropwise until both solids dissolved completely. More water was added (up to 12 ml) until no more precipitate was formed. This was dissolved by heating and allowed to crystallize, then filtered, washed with water, and dried at 110° over P_2O_5 in a vacuum. The yield varied but gave a maximum of 70% at a 2:1 imipramine to ATP molar ratio. The complex could be recrystallized from water without change. The infrared spectrum of the complex (Fig. 3) is

TABLE 1

Drug to 5-HT molar ratios necessary to liberate 50% of bound 5-HT

A 1:2 5-HT–ATP solution containing 5 mM 5-HT was prepared and titrated spectrofluorometrically as described under MATERIALS AND METHODS. As 5-HT is liberated by the drug, the fluorescence emission increases (Fig. 2). ED_{50} values (expressed as drug to 5-HT molar ratio liberating 50% of bound 5-HT) were determined graphically from the titration curves of Fig. 2. Mg^{++} as $MgCl_2$ was equimolar to the ATP used.

Drug	ED_{50}	ED_{50} with Mg^{++}
Amitriptyline	4.5 ± 0.3	7.8 ± 0.4
Nortriptyline	4.1 ± 0.3	4.4 ± 0.2
Imipramine	3.7 ± 0.2	3.4 ± 0.2
Desipramine	2.4 ± 0.2	2.0 ± 0.1
Chlorimipramine	1.6 ± 0.1	1.6 ± 0.1
G 31406	0.5 ± 0.05	0.4 ± 0.05

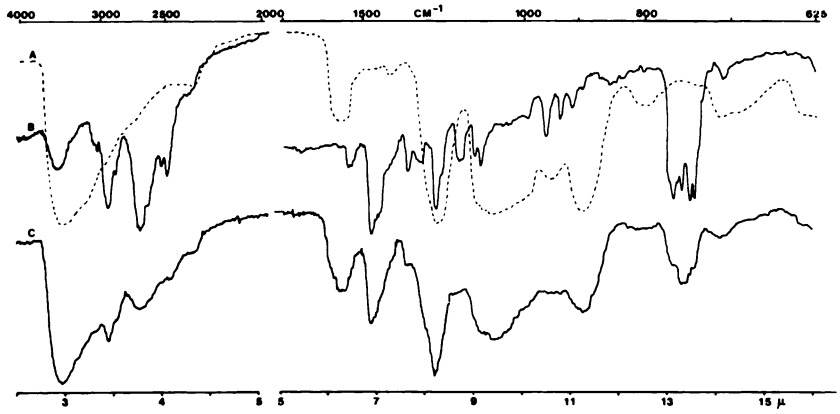

FIG. 3. *Infrared spectra of ATP (A), imipramine HCl (B), and a crystalline 2:1 imipramine–ATP·2Na complex (C) recorded in KBr pellets*

a superposition of the spectra of imipramine and ATP.

Elemental analysis for $C_{48}H_{62}N_9O_{13}P_3Na_2$, corresponding to a 2:1 imipramine-ATP complex, gave the following results.

Calculated: C 51.85, H 5.80, N 11.33, P 8.36, Na 4.12
Found: C 51.38, H 6.37, N 11.42 P 8.39, Na 4.09

Although the yield changed, the composition of the complex was the same regardless of the imipramine to ATP molar ratio.

Complexes prepared from a mixture of 5-HT oxalate, ATP·2Na, and imipramine in a ratio of 2:1:2–4 were formed with a yield of only 25–48%. Complexes from dilute solutions (10–12 mg/ml total) showed spectra identical with those obtained in the absence of 5-HT. At a higher concentration (20–25 mg/ml) some 5-HT seemed to be coprecipitated, as revealed by the phenyl bending of 5-HT at 13.95 μm in the infrared spectrum. Complexes obtained from a 2:1:2 5-HT–ATP–imipramine mixture gave a 2:1 imipramine-ATP complex. By using 4 moles of imipramine we obtained a mixture of a 2:1 and a 3:1 complex as judged from elemental analyses. The composition of this mixture changed upon recrystallization and became a 2:1 complex.

The other thymoleptic drugs investigated (amitriptyline, nortriptyline, chlorimipramine, desipramine, and G 31406) showed similar behavior. In every case we obtained a 2:1 drug-ATP complex, regardless of the presence or absence of 5-HT. However, they were amorphous, noncrystalline solids which dissolved in hot water and separated on cooling. In all cases they gave the correct analyses for a 2:1 complex and displayed the expected spectra.

NMR data. We investigated in detail only the interaction of imipramine with ATP, using the same techniques as employed in our previous work on the 5-HT–ATP interaction (17). Line widths and chemical shifts of the phenyl, C-10–11 bridge, and side chain *N*-methyl protons of imipramine were measured in solutions containing 75 mM drug and 18.7–450 mM ATP (0.25–6 ATP to drug molar ratio). Since conditions of fast ligand exchange were unlikely to be fulfilled, spin-spin relaxation rates were calculated only as relative rates, T_2 obs/T_2 free, according to the method of Hammes and Tallman (20). This gives a relative measure of the immobilization of different functional groups of the molecule. Strong interaction of the side chain (involved in the primary salt bond) and lesser interaction of the dibenzoazepine ring are evident from Fig. 4.

Chemical shift changes of the phenyl and bridge protons are shown in Fig. 5. They indicate strong ring current interaction of the phenyl protons, reflected to a lesser extent by the bridge protons. The *N*-methyl protons did not shift at all.

DISCUSSION

As indicated under INTRODUCTION, there is some experimental evidence of interaction between antidepressants and intraneuronal storage forms of neurotransmitters. Such interactions seem to take the form of interference with the granular uptake as well as release of neurotransmitters, and perhaps also with extragranular storage. It is interesting to recall here the suggestion of Elmquist *et al.* (21), who speculated on a "packaging failure," i.e., insufficient accumulation of transmitter by vesicles, in some forms of myoneural disease.

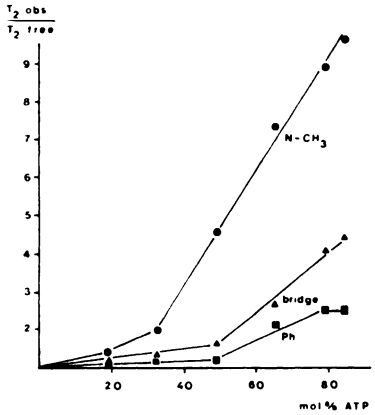

FIG. 4. *Relative NMR relaxation rates of imipramine protons in the presence of varying amounts of ATP*

T_2 obs/T_2 free is plotted for the phenyl, C-10–11 bridge, and *N*-methyl protons, using a 75 mM imipramine HCl solution. Slopes directly proportional to immobilization of the drug bound to ATP.

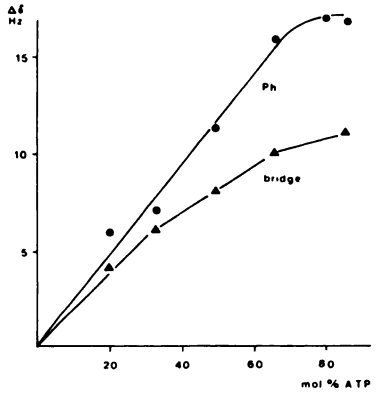

FIG. 5. *Change of NMR chemical shifts of imipramine protons in the presence of ATP*

The upfield chemical shifts of phenyl and C-10–11 bridge protons are shown as a function of mole percentage of ATP. The imipramine HCl concentration was 75 mM.

Addition of drug to a 5-HT–ATP complex solution of constant composition containing 5 mM 5-HT and 10 mM ATP resulted in fluorescence enhancement proportional to the added drug and reached the fluorescence of pure 5-HT (Fig. 2). ATP is nonfluorescent under the experimental conditions used, and corrections were applied to eliminate the inner filter effect of the drug. The fluorescence titration quantitates the efficacy of drugs in competing with serotonin for ATP in this model system. As shown in Table 1, amitriptyline and nortriptyline are the least active, requiring over 4 moles/mole of total 5-HT to replace 50% of the bound serotonin. Since in the 1:2 5-HT–ATP solution only about 50% of the 5-HT is bound, this ultimately means that somewhat more than 2 moles of these drugs replace 1 mole of 5-HT in the complex. Since the drug will bind with the free ATP as well, a very complex equilibrium mixture results and absolute numbers may be impossible to calculate. Imipramine is somewhat more active, desipramine slightly more so. Chlorimipramine, one of the clinically most active drugs, has an ED_{50} value of 1.6. The 10,11-dehydroimipramine derivative G 31406, an experimental drug, is the most active, 0.5 mole replacing 50% 5-HT.

The order of potency for displacing 5-HT from the 5-HT–ATP complex *in vitro* found in our experiments (Table 1) is comparable only in part with the reported efficacy of tricyclic antidepressants in inhibiting the synaptosomal uptake of 5-HT (22, 23). However, the high potency of chlorimipramine in displacing 5-HT is in good agreement with the ability of this drug to block neuronal uptake of 5-HT selectively. It is of interest that this drug has been found to cause release of 5-HT from brain synaptosomes (9, 23).

The addition of $MgCl_2$ has a marginal effect on ED_{50} values except for amitriptyline, when it rises to 7.8. No explanation for this can be offered at the present time.

The observation of the formation of insoluble complexes from all the drugs and ATP in the absence and presence of 5-HT further demonstrates competition between drug and 5-HT for ATP. Analogous insoluble complexes formed between norepinephrine and ATP were described by Maynert and co-workers (24, 25). These authors came to much the same conclusions as the Pletscher group and ourselves: that colloidal micelles or, even better, insoluble solids would fulfill admirably the role of a nondiffusible and nonhypertonic storage complex of neurotransmitters.

While precipitation of a saltlike drug-ATP complex does not necessarily mean a higher stability constant than that of 5-HT–ATP, and insoluble salt formation of large cations and anions is not entirely surprising, simple coprecipitation can be ruled out. The concentration of components in the solution is much below their solubility limit (except for 5-HT); salting out is therefore unlikely. In addition, the complexes can be repeatedly recrystallized or reprecipitated from hot water in an unchanged molar ratio, which would not be possible with a noninteracting mixture.

Interpretation of NMR data for the imipramine-ATP interaction are also in agreement with the foregoing conclusions. The plot of T_2 obs/T_2 free vs. mole percentage of ATP (Fig. 4) demonstrates that the side chain is highly immobilized (as shown by the steep line width change of the *N*-methyl proton singlet of imipramine), followed by the immobilization of the C-10–11 bridge protons, and finally that of the slightly immobilized phenyl protons. This

is similar to our results published on the 5-HT–ATP interaction (17), which showed that the complex is held together by ionic bonds assisted by weak ring current binding. Ring currents are manifested in upfield shifts of resonance lines. When the change in chemical shifts is plotted against mole percentage of ATP (Fig. 5) a steep change for the phenyl protons is seen, with a lesser shifting of bridge protons; the latter, of course, is only a reflection of the change in the shielding by the phenyls. The *N*-methyl protons of the side chain showed no change, as expected. A Foster-Fyfe plot (26) for the bridge protons could be constructed and is reasonably linear (Fig. 6), but was not used to calculate an association constant because of suspected slow exchange rates. Nevertheless, it is additional proof of binding analogous to the 5-HT–ATP case (17).

The concentrations of serotonin, ATP, and drugs, even in the fluorescence titration, are relatively high. It has to be kept in mind, however, that high local neurotransmitter and ATP concentrations are quite likely in storage forms, whether granular or otherwise. For instance, it has been calculated that the concentration of norepinephrine in the vesicles lies between 0.9 and 2.4 M (27). We observed (18) that no fluorescence quenching of 5-HT by ATP could be demonstrated below 0.1 mM 5-HT, since the complex did not form. In terms of transmitter release this is, of course, quite reasonable; upon quantal expulsion of the 5-HT–ATP "package" by exocytosis (28) the complex is diluted in the synaptic gap and liberates free 5-HT.

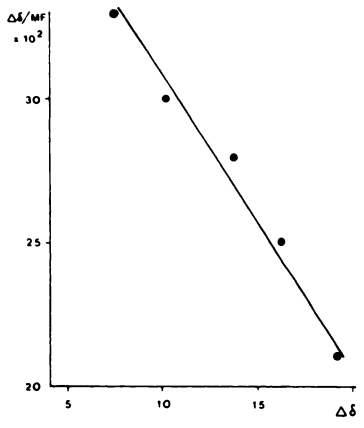

FIG. 6. *Foster-Fyfe plot of imipramine C-10–11 bridge protons*

Chemical shift change (Δδ)/mole fraction (MF) of ATP is plotted against Δδ. Linearity proves ring current interaction but is not suitable for association constant calculation.

The high concentration used in the crystalline drug-ATP complex formation experiments are of preparative significance only, and crystalline complex formation *in vivo* is not suggested. Similarly, the concentrations used in the NMR experiments are only a technical necessity.

The use in our model of drug concentrations above those that occur in tissues after therapeutic doses does not preclude a hypothetical extrapolation of our results *in vitro* to living systems. Drug to 5-HT molar ratios of 0.5—4.5 do not seem to be unattainable in a synapse, in view of the minute amounts of neurotransmitter present, and only the relative ratios are of significance. Transport of drug into storage vesicles does not necessarily have to be involved to produce increased 5-HT levels intraneuronally; inhibition of "repackaging" or interference with extragranular storage might be considered.

The validity of our hypothesis on intraneuronal interference of antidepressants with 5-HT storage, based on experiments *in vitro,* awaits further confirmation in a living system.

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