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Uptake of 5-hydroxytryptamine in blood platelets and its inhibition by drugs: role of plasma membrane and granular storage

ANDREAS LAUBSCHER AND ALFRED PLETSCHER*

Research Division, F. Hoffmann-La Roche & Co. Ltd., 4002 Basel, Switzerland

The initial uptake of ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) showed linearity for short time intervals in normal and reserpinized blood platelets of guinea-pigs, but was lower in reserpinized platelets. The K_m values for the ^{3}H-5-HT uptake were virtually identical in normal and reserpinized platelets, whereas V_{max} was lower in the latter. Imipramine and chlorpromazine caused the same percentage inhibition of ^{3}H-5-HT uptake in normal and reserpinized platelets; the reserpine-like compound Ro 4–1284 inhibited the uptake of ^{3}H-5-HT in the normal, but not markedly in the reserpinized platelets. Haloperidol, prenylamine and Ro 4–9040 were more potent inhibitors in normal than in reserpinized platelets. It is concluded that (a) the K_m of the initial uptake of 5-HT by platelets is probably determined by the mechanism at the plasma membrane, whereas V_{max} may be codetermined by the intracellular storage capacity, (b) platelets are models for differentiating the site of action (plasma membrane or storage organelles) of drugs interfering with 5-HT uptake, and (c) neuroleptics- and reserpine-like compounds may either act selectively on the plasma membrane or on the intracellular storage organelles, or affect both of these subcellular sites.

Blood platelets of various species take up 5-hydroxytryptamine (5-HT) by a specific, carrier-mediated mechanism which operates at low concentrations (of the amine in the medium) and is located at the level of the plasma membrane (Sneddon 1973; Rudnick 1977; Pletscher 1978). Part of the 5-HT taken up by the membrane mechanism is stored in specific intracellular organelles (5-HT storage granules) by a process not yet fully elucidated. Various psychotropic drugs inhibit the 5-HT uptake by normal platelets (Sneddon 1973; Pletscher 1978). Imipramine has been demonstrated to exert its inhibitory action preferentially at the plasma membrane (Da Prada & Pletscher 1968; Reimers et al 1977; Rudnick 1977), whereas reserpine abolishes the uptake of the amine into the 5-HT storage organelles (Da Prada & Pletscher 1968; Costa et al 1977) without interfering to a major extent with its uptake into the cytoplasm (Costa et al 1975). It remains to be shown whether the plasma membrane alone is responsible for the uptake kinetics of 5-HT in normal platelets or whether the intracellular 5-HT storage process plays a role, too. Furthermore, it is not clear whether most inhibitors of 5-HT uptake exert their action preferentially at one site, i.e. at the plasma membrane or at the storage organelles, or at both.

* Correspondence and present address: Department of Research, Kantonsspital, Hebelstrasse 20, CH 4031 Basel, Switzerland.

To clarify these questions, the uptake kinetics of ^{3}H-5-HT have been compared in normal and reserpinized platelets of guinea-pigs which resemble those of man in the uptake of 5-HT (preliminary results of this laboratory), 5-HT content and number of highly dense osmiophilic 5-HT storage organelles (Tranzer et al 1966). In addition, a relatively simple procedure to determine the site of action of 5-HT uptake inhibitors in platelets is described.

MATERIALS AND METHODS

Materials

The [1,2^{3}H]-5-hydroxytryptamine creatinine suphate (specific radioactivity 26 Ci mmol^{-1}) (^{3}H-5-HT) was obtained from New England Nuclear Corporation and the unlabelled 5-HT from Fluka AG (Buchs, Switzerland). In addition, the following substances were used: reserpine, imipramine (Ciba-Geigy Ltd., Basel), chlorpromazine, prenylamine, haloperidol, Ro 4–1284 (2-hydroxy-4-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexyhydro-11bH-benzo[a] quinolizine HCl), Ro 4–9040 bis (3,4-dichlorophenethyl)-amine (all from F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland) and nialamide (Pfizer, U.S.A.). Stractan (polyarabinogalactane, mol. wt about 30 000) was purchased from St. Regis Libby, Montana, U.S.A., purified and prepared according to previously described methods (Corash et al 1974,

1977; Graf et al 1978), without the addition of bovine serum albumin.

Platelet isolation

Female guinea-pigs (Himalayan spotted, 600–800 g) were bled under light ether anesthesia through a polyethylene cannula inserted in a carotid artery. Some animals were treated with reserpine (5 mg kg^{-1}, i.p.) 16 h before bleeding. The whole blood was collected in a plastic vial and mixed with 1/10 vol 3·8% trisodium citrate. $2H_2O$. Platelet-rich plasma (PRP) was prepared by centrifugation of the whole blood at 400 *g* for 10 min in plastic tubes. PRP from 1–4 animals was pooled and each pool halved.

Reserpine (10^{-3} M in 0·6% glacial acetic acid; final concentration 2×10^{-6} M), was added to one half and the solvent alone to the other. Both were then shaken gently at 37 °C for 30 min. Thereafter, in some of the experiments, the PRP (with and without addition of reserpine) was put on a density gradient consisting of 7 ml 10% stractan solution layered on 5 ml 20% stractan. The tube (cellulose nitrate, 8·75 × 2·5 cm) was centrifuged for 10 min at 4 °C and 7000 *g* in a Sorvall centrifuge with a swing-out rotor. The platelets which assembled in a 2–4 mm layer above the 20% stractan solution were removed by puncturing the bottom of the tube with a hollow needle. Platelet recovery was 60–70% compared with the PRP. The concentrated platelet suspension was diluted with 5 vol of Tris buffer (tris: g $litre^{-1}$ NaCl 8·21, KCl 0·57, glucose 1·01, Tris (hydroxymethyl)-aminomethane 0·93, Trisodium citrate . $2H_2O$ 3·8.), pH 7·4. Platelets were then counted in a Coulter counter and the platelet concentration adjusted to 10^5 μl^{-1} by dilution with Tris. In other experiments, PRP (with and without reserpine) was diluted with Tris to give the same final platelet concentration.

Uptake experiments

Aliquots (1 ml) of the final platelet suspensions were preincubated at 37 °C for 30 min under gentle shaking. Tris (0·05 ml) either alone (controls), or containing various concentrations of imipramine, neuroleptics, reserpine-like drugs, or nialamide (final concentrations 10^{-4} M) was added 10 min before the end of the preincubation period. Then the mixture was supplemented with 0·05 ml ^{3}H-5-HT creatinine sulphate in 10^{-4} M HCl. Incubation was stopped after various times (time curves), after 1 min (uptake kinetics) or after 5 min (inhibitor experiments) by adding 3 ml of formaldehyde (2%) (Costa & Murphy 1975). Platelets were then separated on a Millipore filter (cellulose nitrate, diameter 2·5 cm, pore size 0·45 μM) under vacuum. Samples were washed twice with 5 ml Tris. Unspecific binding of ^{3}H-5-HT was estimated by incubating platelets with ^{3}H-5-HT and 1000-fold excess of the unlabelled amine and submitting them to the same procedure. Filters were dried, put into counting vials, and the amount of radioactivity was measured in a scintillation counter. Protein determination was carried out according to Lowry et al (1951) in aliquots of the final platelet suspension.

The measurements of the time curves for ^{3}H-5-HT uptake were carried out with platelets supended in plasma or with platelets isolated by a stractan gradient and incubated in Tris. The inhibitor experiments were all performed with stractan-isolated platelets in order to avoid errors due to protein binding.

Calculations

In all experiments, the non-saturable uptake was subtracted from the total uptake. The K_m and V_{max} values were obtained by applying the least square non-linear regression method. The IC50 values (concentrations in μM causing half maximal inhibition of ^{3}H-5-HT uptake) were determined graphically. Statistical analysis was performed with Student's *t*-test or the Wilcoxon signed-ranks test (two sided) (Hollander & Wolfe 1973).

RESULTS

5-HT uptake by normal platelets

In normal platelets incubated in plasma with 10^{-7} or 10^{-6} M ^{3}H-5-HT, the uptake of this amine was linear for at least 5 min (Fig. 1). With rising concentrations of ^{3}H-5-HT in the incubation medium the uptake tended towards a saturation level, which was reached at about 2×10^{-6} M ^{3}H-5-HT. The K_m and V_{max} values after incubation for 1 min are indicated in Table 1. The uptake curves for platelets isolated by a stractan gradient and incubated in Tris were similar to those for platelets incubated in plasma.

5-HT uptake by reserpinized platelets

Platelets preincubated in plasma with reserpine took up significantly less ^{3}H-5-HT than platelets not so preincubated ($P < 0{\cdot}01$ or $< 0{\cdot}05$) at all time intervals tested (Fig. 1). The maximum effect was reached after less than 10 min of incubation with the drug. Rising concentrations of reserpine caused an increasing inhibition of the ^{3}H-5-HT uptake, maximal effects (about 60% decrease in uptake after 10 min incubation) being reached between 10^{-7} M and 10^{-6} M.

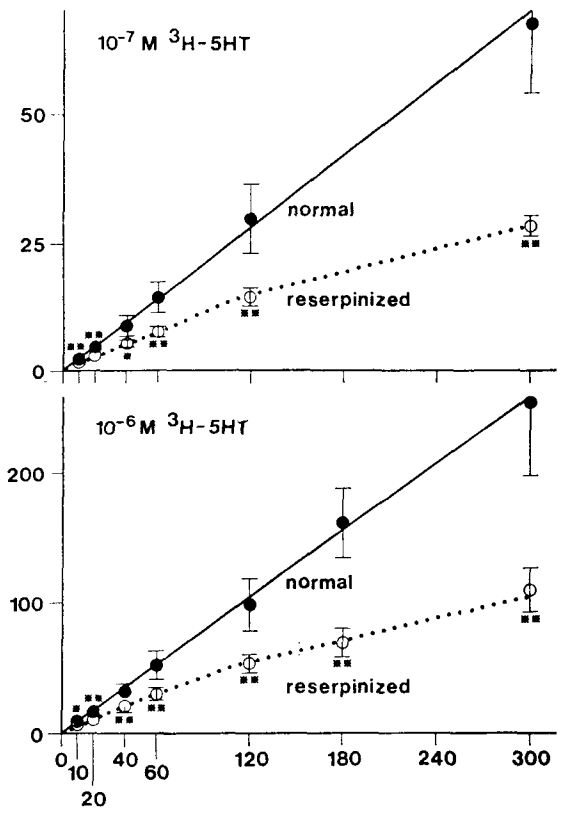

FIG. 1. Uptake of ^{3}H-5-hydroxytryptamine (^{3}H-5-HT, ordinate: pmol mg^{-1} protein) by normal and reserpinized platelets incubated at 37 °C in plasma for various times. Initial concentrations of ^{3}H-5-HT in the plasma: 10^{-7} and 10^{-6} M. The values are averages with s.e.m. obtained from 6–8 experiments each with platelet-rich plasma (PRP) from 1–2 animals. Abscissa: time (s). * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$, Wilcoxon test.

On incubation with very high concentrations (5×10^{-5} M) an abrupt further decrease in the platelet 5-HT uptake occurred, which was possibly due to an unspecific impairment of platelet function.

For the first minute the uptake of ^{3}H-5-HT by platelets preincubated with 2×10^{-6} M reserpine showed linearity, but then the rate of uptake decreased (Fig. 1). Since the uptake by normal platelets was linear for at least 5 min, the ratio of labelled amine taken up by reserpinized platelets to that accumulated by normal platelets was lower after 5 min (about 40%) than after 1 min (55%).

With increasing concentrations of ^{3}H-5-HT in the incubation medium, the uptake of the amine tended towards a saturation level. The K_m of the ^{3}H-5-HT uptake measured after 1 min was the same as that found in normal platelets, whereas V_{max} was significantly lower in reserpinized than in normal platelets ($P < 0{\cdot}01$) (Table 1).

Table 1. K_m and V_{max} of normal and reserpinized platelets incubated for 1 min at 37 °C in plasma with various concentrations (7×10^{-8} to 10^{-6} M) ^{3}H-5-hydroxytryptamine (^{3}H-5-HT). The values are averages with s.e.m. obtained from 4 experiments each performed with pooled platelets from 2 animals. V_{max} and K_m are indicated in pmol mg^{-1} protein min^{-1} and μM, respectively.

	K_m	V_{max}
Normal	0·44 ± 0·02	74·0 ± 1·7
Reserpinized	0·47 ± 0·06	40·4 ± 2·5

Significance (normal versus reserpinized, Students t-test): K_m: $P > 0{\cdot}05$; V_{max}: $P < 0{\cdot}01$.

Platelets which were preincubated with reserpine in plasma, isolated by a stractan gradient, and then incubated with ^{3}H-5-HT behaved similarly to platelets incubated with ^{3}H-5-HT in plasma containing the reserpine. Platelets isolated from animals injected with reserpine (5 mg kg^{-1}, i.p.) 16 h before bleeding, showed a ^{3}H-5-HT uptake curve similar to platelets from untreated animals preincubated with 2×10^{-6} M reserpine. Addition of a monoamine oxidase (MAO) inhibitor (nialamide 10^{-4} M) to the incubation media of normal and reserpinized platelets did not have any relevant influence on the ^{3}H-5-HT uptake curves.

Imipramine and Ro 4–1284

The antidepressant imipramine caused a concentration-dependent decrease of the ^{3}H-5-HT uptake in both normal and reserpinized platelets during the first 5 min of incubation, i.e. when ^{3}H-5-HT uptake in normal platelets is linear. The IC50 of imipramine as well as the shapes and the slopes of the inhibition curves (calculated in per cent of the uptake by the corresponding platelets without addition of imipramine) were the same for normal (N) and reserpinized (R) platelets (Fig. 2, Table 2). Therefore, the quotient IC50R/IC50N was close to 1. The reserpine-like benzoquinolizine Ro 4–1284 also induced a concentration-dependent decrease of the ^{3}H-5-HT uptake in normal platelets, the IC50 being 75 nM. As with reserpine, the 5-HT uptake of the platelets did not decrease below about 40% of that of normal platelets. In reserpinized platelets, Ro 4–1284 in concentrations up to 10 μM only slightly diminished the ^{3}H-5-HT uptake (at the most by about 8%). Therefore an IC50 could not be determined (IC50 $> 10^{-5}$ M) (Fig. 2, Table 2).

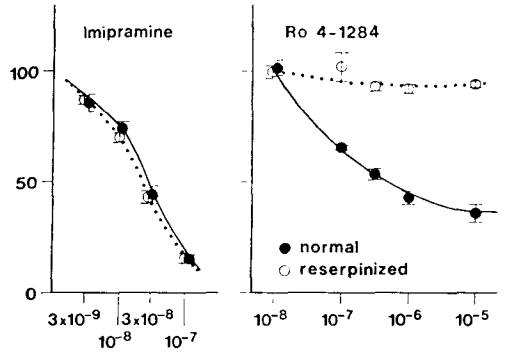

FIG. 2. Effect of various concentrations of imipramine and Ro 4–1284 on the ^{3}H-5-HT uptake (ordinate: ^{3}H-5-HT uptake in %) by normal and reserpinized platelets isolated with a stractan gradient. The ^{3}H-5-HT uptake was calculated as per cent of that occurring in normal and reserpinized platelets without addition of the inhibitors. The values are averages with s.e.m. obtained from 4 experiments each performed with pooled platelets from 4 animals. Significance (reserpinized versus corresponding normal platelets, Student's *t*-test): Abscissa: concentration of inhibitor (M). Imipramine: $P > 0{\cdot}05$ (all values); Ro4–1284: $P < 0{\cdot}01$ (all values except 10^{-8} M: $P > 0{\cdot}05$).

Table 2. Modification of the IC50 of imipramine by Ro 4–1284 and of the IC50 of Ro 4–1284 by imipramine in normal and reserpinized platelets.

IC50 means concentration of drugs causing half maximal inhibition of uptake of ^{3}H-5-HT (initial concentration 10^{-7} M). Stractan-isolated platelets. The ratios imipramine: Ro 4–1284 are based on the IC50 values of the compounds. As an example, a ratio imipramine: Ro 4–1284 of 1 : 10 means 26 nmol imipramine (value of the IC50 of imipramine): 740 nmol Ro 4–1284 (=10 times the value of the IC50 of Ro 4–1284). The values are averages with s.e.m. obtained from 3–4 experiments each performed with pooled platelets from 4 animals.

Drug ratios	Normal (N)	Reserpinized (R)	IC50R/ IC50N
	IC50 imipramine		
Imipramine : Ro 4–1284			
1:0	25·5 ±2·7	23·4 ±2·6	0·9 ±0·1**
1:3·5	11·2 ±0·9*	27·2 ±6·1	2·4 ±0·3**
1:10	3·3 ±1·1*	21·1 ±3·2	7·3 ±1·3**
1:30	1·6 ±0·4*	18·5 ±2·6	12·1 ±1·5**
	IC50 Ro 4–1284		
Ro 4–1284 : Imipramine			
1:0	75·0 ±6·7	>100000	>100
1:0·03	54·0 ±10·5	620·0 ±143·0	11·4 ±0·9
1:0·10	41·3 ±8·8*	226·7 ±66·9	5·8 ±1·5†
1:0·30	28·0 ±4·0*	82·5 ±27·5†	2·9 ±0·6†

* *P* versus imipramine or Ro 4–1284 alone: <0·01 (Students *t*-test).

** All values significantly different from each other ($P < 0{\cdot}01$).

† Significantly different from Ro 4–1284 + imipramine 1:0·03 ($P < 0{\cdot}01$).

When Ro 4–1284 was added to imipramine, in increasing proportions based on the IC50 of the compounds (see Table 2), the IC50 of imipramine gradually decreased in normal platelets, whereas in reserpinized platelets it was only slightly but not significantly reduced ($P > 0{\cdot}05$). As a consequence, the quotient IC50R/IC50N of imipramine increased. Conversely, the quotient IC50R/IC50N of Ro 4–1284 decreased when imipramine was added in increasing ratios to Ro 4–1284 (Table 2).

Other drugs

The concentration-dependent inhibition of ^{3}H-5-HT uptake by the neuroleptic chlorpromazine was the same for normal and for reserpinized platelets, the quotient IC50R/IC50N being 1 like that of imipramine. Haloperidol in all concentrations tested, inhibited ^{3}H-5-HT uptake more strongly in normal than in reserpinized platelets. The quotient IC50R/IC50N was about 2. The reserpine-like drugs prenylamine and Ro 4–9040 also showed an inhibitory action in both normal and reserpinized platelets; however, the drugs, in all inhibitory concentrations tested, were more potent in the former than in the latter, the quotient IC50R/IC50N being about 6 for prenylamine and about 9·5 for Ro 4–9040 (Table 3).

Table 3. IC50 in nM of various drugs in normal and reserpinized platelets. IC50 means concentrations of drugs causing half maximal inhibition of uptake of ^{3}H-5-HT (initial concentration 10^{-7} M). Stractan isolated platelets. The values are averages with s.e.m. obtained from 3–4 experiments each performed with pooled platelets from 2 animals.

Drug	Normal (N)	Reserpinized (R)	R/N
Chlorpromazine	144·7 ± 38·1	145·9 ± 19·8	1·1 ± 0·1
Haloperidol	1145·5 ± 74·6	2406·3 ± 150·4	2·1 ± 0·2*
Prenylamine	184·4 ± 21·3	1072·8 ± 66·8	6·1 ± 1·1*
Ro 4–9040	78·4 ± 14·7	699·0 ± 41·1	9·4 ± 1·2*

* *P* versus chlorpromazine and imipramine: <0·01 (Students *t*-test).

DISCUSSION

The K_m values of the ^{3}H-5-HT uptake were virtually identical in normal and in reserpinized platelets of guinea-pigs up to 1 min after the start of the incubation, when the uptake was still linear in both types of platelets. This indicates that the K_m is not markedly influenced by the intracellular 5-HT storage mechanism, but depends rather on the transport process at the plasma membrane.

On the other hand, the uptake of ^{3}H-5-HT and V_{max} measured during the initial linear phase were significantly lower in reserpinized than in normal platelets, indicating that V_{max} not only depends on the mechanism at the plasma membrane, but also on the intracellular storage process. The enhanced metabolism of ^{3}H-5-HT occurring in reserpinized platelets (Pletscher et al 1967) is unlikely to be the reason for the decreased ^{3}H-5-HT accumulation, since the MAO inhibitor nialamide did not cause any relevant change in the ^{3}H-5-HT uptake curves. Furthermore, it is not likely that the light inhibition by reserpine of the passive outflow of 5-HT from platelets (Bartholini et al 1965) caused the marked decrease in V_{max} in reserpinized platelets. An inhibiting effect on passive outflow would, if anything, probably rather tend to increase V_{max}.

The initial ^{3}H-5-HT uptake was also lower in reserpinized than in normal platelets of man and rabbits (results not shown).

The present results are in partial disagreement with earlier experiments, in which no differences between the initial ^{3}H-5-HT uptakes in normal and reserpinized platelets of rabbits and man could be found (Reimers et al 1977; Stahl & Meltzer 1978). The reason for this is not clear, but might be connected with methodological differences. Thus, the present experiments use PRP or platelets isolated by a stractan gradient, instead of washed platelets suspended in artificial buffers as in previous work. However, our experiments do not completely exclude the possibility that ^{3}H-5-HT uptake is the same in reserpinized and normal platelets at time intervals of up to about 10 s and that right at the beginning it does not depend on the intracellular storage of the amine. Despite this possibility, the present experiments clearly show that at time intervals after the first 10 s of the incubation, when uptake experiments are usually performed, reserpine causes disturbances of the intracellular 5-HT storage process, reflected by a decreased V_{max}.

These findings are of importance with regard to inhibitors of 5-HT uptake. The use of both normal and reserpinized platelets, in which uptake is abolished at the storage granules but not at the plasma membrane (Da Prada & Pletscher 1968; Costa et al 1975, 1977), can show whether an inhibitor of 5-HT uptake has its principle site of action at the plasma membrane or at the intracellular storage organelles. In fact, imipramine, which acts preferentially at the plasma membrane (Reimers et al 1977), caused the same percentage inhibition of ^{3}H-5-HT uptake in normal and in reserpinized platelets at all the inhibitor concentrations used (identical concentration-inhibition curves). Thus, the quotient IC50R/IC50N of the drug was close to 1. It is however possible that very high concentrations of imipramine (much higher than the IC50) have an effect on the intracellular 5-HT storage, and this has indeed been previously demonstrated (Bartholini & Pletscher 1961).

On the other hand, Ro 4–1284, which preferentially inhibits 5-HT uptake at the granular 5-HT storage sites (Pletscher et al 1962; Da Prada et al 1967), interferred with 5-HT uptake much less in reserpinized platelets than in normal, the quotient IC50R/IC50N being far above 100.

It is therefore to be expected that drugs with a quotient IC50R/IC50N above 1 and below 100 exert a double action, i.e. on both the plasma membrane and on the 5-HT organelles. This could be demonstrated by using mixtures of imipramine and Ro 4–1284. Table 2 indicates that the IC50R/IC50N of imipramine rose when Ro 4–1284 was added to it in increasing proportions, whereas the IC50R/IC50N of Ro 4–1284 decreased with increasing additions of imipramine. To change the IC50 of the membrane inhibitor imipramine, the granular inhibitor Ro 4–1284 had to be added in relatively high amounts, whereas the IC50 of Ro 4–1284 was changed by addition of relatively little imipramine. This finding is probably at least partly connected with the fact that imipramine interferes with the first step of the ^{3}H-5-HT uptake, i.e. the transport of the amine at the plasma membrane, whereas Ro 4–1284 acts on the second step, i.e. the incorporation of the intracellular, extra-granular ^{3}H-5-HT into the storage organelles. Preliminary experiments showed that an interference by imipramine with the entry of Ro 4–1284 into the platelets is unlikely.

One of the two neuroleptic drugs tested, chlorpromazine, exhibited the same pattern of activity as imipramine in normal and reserpinized platelets (identical concentration-inhibition curves in both types of platelets, quotient IC50R/IC50N of about 1). Therefore, chlorpromazine probably acts primarily on the plasma membrane. The other, haloperidol, probably acts at both sites, since at all concentrations tested the drug was more potent in normal than in reserpinized platelets, leading to a quotient IC50R/IC50N of more than one. These results show that neuroleptic compounds do not all have the same selectivity in their action on the plasma membrane, and that some of them (e.g. haloperidol) simultaneously affect the intracellular amine storage. A possible action of haloperidol on the granular

storage of other amines, e.g. of dopamine in the striatum, might explain why this neuroleptic impairs extrapyramidal motoricity more markedly than chlorpromazine. On the other hand, prenylamine and Ro 4–9040, two drugs thought to exert a reserpine-like action (Juorio & Vogt 1965; Pletscher et al 1965) also showed a quotient IC50R/IC50N markedly above 1 but considerably below 100, indicating an action on the plasma membrane as well as on granular storage. However, an exact quantitative estimation of the membrane and the granular components of an inhibitor of 5-HT uptake is not possible. The quotient IC50R/IC50N has to be rather considered as a semiquantitative index.

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