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THE USE OF THIOPROPERAZINE, A PHENOTHIAZINE DERIVATIVE, AS A LIGAND FOR NEUROLEPTIC RECEPTORS—I.

IN VITRO STUDIES

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Abstract—The use of [^{3}H]thioproperazine (a neuroleptic of the phenothiazine family) as a ligand for neuroleptic receptors has been studied *in vitro* in various brain regions of the rat. Specific thioproperazine binding has been observed in two regions rich in dopamine terminals: striatum and nucleus accumbens. No specific binding was observed in frontal cortex and hippocampus. Thioproperazine binds with high affinity (K_d = 0.30 nM) to rat microsomal striatal fraction. In this fraction the number of binding sites (1.47 pmoles/mg protein) is rather similar to that found with haloperidol and spiperone, two neuroleptics of the butyrophenone type. Subcellular distribution of thioproperazine binding reveals a pattern similar to that of 5′-nucleotidase, a plasma membrane marker. This indicates that the receptor sites of [^{3}H]thioproperazine are associated with membrane-like structures. Inhibition studies indicate that all neuroleptic compounds, belonging to different chemical families, are capable of displacing thioproperazine from its binding sites. The most potent drugs are spiperone, thioproperazine and pipotiazine. Dopamine and the two dopamine agonists, apomorphine and bromocryptine, present affinity for thioproperazine binding sites. It is concluded that thioproperazine, a phenothiazine neuroleptic, can be used, like the butyrophenone derivatives haloperidol and spiperone, as a ligand for studying neuroleptic receptors.

Many ligands have been used to identify the dopamine receptors in the brain. Dopamine [1] and apomorphine [2] served first to identify the agonist state of the dopamine receptors, the antagonist state being labelled by neuroleptics [3]. Otherwise it has been proposed that dopamine binds to pre-synaptic sites and neuroleptics to post-synaptic sites [4].

Many neuroleptics were tested as ligands for dopamine receptors, the most commonly used being the two butyrophenone derivatives haloperidol and spiperone [3, 5]. However, other neuroleptics such as clozapine [6] or *cis*-flupentixol [7] and more recently, an antagonist of dopamine with potent antiemetic activity, domperidone [8], have been tested as ligands for dopamine receptors.

Chlorpromazine, which has been the first drug found active in psychiatric states [9] and remains the reference compound for neuroleptics, is a phenothiazine derivative. However, since the introduction of binding techniques it seems that no publication has appeared about the use of a phenothiazine neuroleptic as a ligand for *in vitro* studies of dopamine receptors. One could thus wonder whether the phenothiazine neuroleptics are unsuitable ligands, or whether their binding sites could be different from those of other neuroleptics.

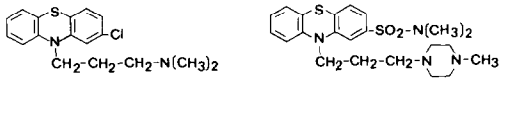

Fig. 1. Chemical structure of chlorpromazine and thioproperazine.

This is not the case, as will be shown in the present paper where we describe the *in vitro* binding properties of thioproperazine (R.P. 7 843), and in the companion paper [10] where we shall study the *in vivo* binding of this compound.

This phenothiazine neuroleptic compound was chosen because of its strong activity in psychiatry [11] and its behavioural and biochemical profile in animal models [11, 12]. Moreover, the selective activity of thioproperazine is revealed in pharmacological tests by its strong activity against apomorphine-induced stereotypies in rats and emesis in dogs and its potent cataleptic effect with weak sedative effects in such tests as barbiturate potentiation and traction.

METHODS

Tissue preparation. Male rats (200 ± 10 g body wt, CD_1, C.O.B.S. Charles River, France) were decapitated and the brain removed and placed on an ice-cold Petri dish. The cerebral structures (striatum, nucleus accumbens, hippocampus or frontal cortex) were dissected and put in ice-cold 0.25 M sucrose containing 3 mM imidazole, pH 7.4. Homogenization was performed in 10 vol. of sucrose, by ten strokes of the tight pestle of a Dounce homogenizer. Tissue fractionation was performed by differential centrifugation using the centrifugal forces described

by Tulkens for fibroblasts [13]. Two different centrifugation procedures were used, in the first four fractions N, M, LP and S were obtained, in the second five fractions N, M, L, P and S were obtained.

Binding studies. The assays were mainly performed on LP and P fractions. For the study of the subcellular distribution of [^{3}H]thioproperazine binding, the other fractions were also tested.

The fractions were diluted in 50 mM Tris buffer, pH 7.7, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline, in order to obtain a final concentration in proteins of 0.5 mg/ml for LP fractions and 0.3 mg/ml for P fractions. The suspension was homogenized with an Ultra Turrax homogenizer at a setting of 7 (full range = 10) for 15 sec and dispersed in polystyrene tubes (1 ml in each tube). These tubes were placed for 5 min in a 37° bath and kept in ice until they were used. Different concentrations (in a volume of 0.1 ml) of [^{3}H]thioproperazine and of unlabelled drugs were added to proteins, then incubation was performed at 37° for 24 min. At the end of the incubation time, 1 ml of the mixture was filtered under suction through GF/B glass fibre filters (Whatman). Filters were washed twice with 5 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.7. The radioactivity of the filters was counted by liquid scintillation spectrometry using a Berthold liquid scintillation spectrometer. The specific binding of thioproperazine was measured as the excess over blanks taken in the presence of 10 μM of unlabelled thioproperazine.

Labelled thioproperazine was diluted in a solution of ascorbic acid (0.1 per cent) and bovine serum albumin (0.6 per cent). The unlabelled drugs were dissolved with 0.1 per cent ascorbic acid and further diluted with the same solution.

The determination of the concentration of the drugs producing 50 per cent inhibition of displaceable binding of the labelled ligand (IC_{50}) was estimated graphically. The percentage of specific bound ligand was plotted vs the log of each drug concentration. The inhibition curves were composed of at least six points.

Biochemical assays. Enzymatic assays were performed as described by Tulkens [13]; the pH of assays was similar to that described for fibroblasts except for 5'-nucleotidase (EC 3.1.3.5) whose optimum pH in striatum is 9. Phosphoglucomutase (EC 2.7.5.1) was assayed at pH 7.0 according to Ray and Roscelli [14]. Proteins were determined by the method of Lowry *et al.* [15].

Materials. [^{3}H]Thioproperazine (sp act. 18.5 Ci/mmole) was prepared by Mr. Raballand (Rhône-Poulenc and C. E. A. Saclay, France). The other drugs were obtained from various pharmaceutical companies. The materials for biochemical assays were of analytical grade, and were purchased from Merck, A. G. (Darmstadt, F.R.G.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Koch-light (Colnbrook, U.K.).

RESULTS

Saturability of [^{3}H]thioproperazine binding. Incubation of a microsomal fraction (fraction P) of rat striatum with various concentrations of [^{3}H]thioproperazine (0.1–2.5 nM) reveals that binding of [^{3}H]thioproperazine is saturable (Fig. 2). The non-specific binding measured in the presence of 10 μM of unlabelled thioproperazine is linear and accounts at half saturation for about 10 per cent of total binding.

Scatchard analysis (Fig. 3) of the specific [^{3}H]thioproperazine binding indicates, for striatum, one high affinity binding site having an apparent dissociation constant of 0.31 nM; the density of receptors is 1.44 pmoles/mg protein. The value of the Hill coefficient is 0.92. On six experiments made independently on the P fraction of rat striatum the mean ± S.E.M. of the dissociation constant is 0.30 ± 0.03 nM, and that of the number of binding sites is 1.47 ± 0.11 pmoles/mg protein.

Kinetics of specific thioproperazine binding. As shown in Fig. 4, the maximum specific binding of [^{3}H]thioproperazine is obtained after an incubation time of 24 min. The dissociation curve was measured by adding a large excess (25,000-fold) of cold ligand after equilibrium was obtained. The release of [^{3}H]thioproperazine is shown in Fig. 5. The dissociation rate of [^{3}H]thioproperazine is 0.028 min^{-1} and $t_{1/2}$ = 25 min. The rate constant for association

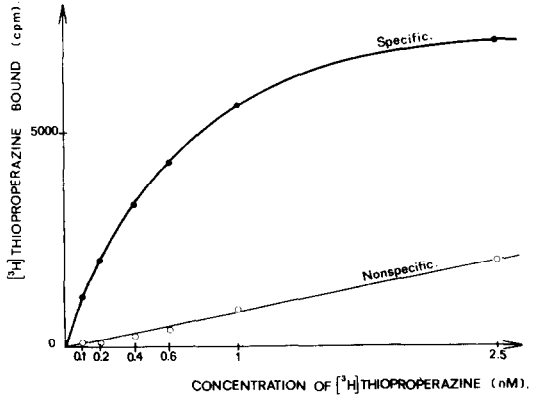

Fig. 2. Binding of [^{3}H]thioproperazine to rat striatum microsomal fraction.

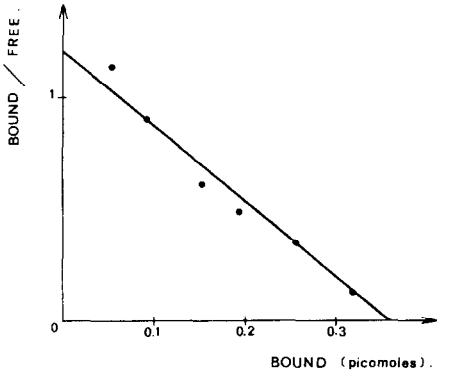

Fig. 3. Scatchard analysis of specific [^{3}H]thioproperazine binding.

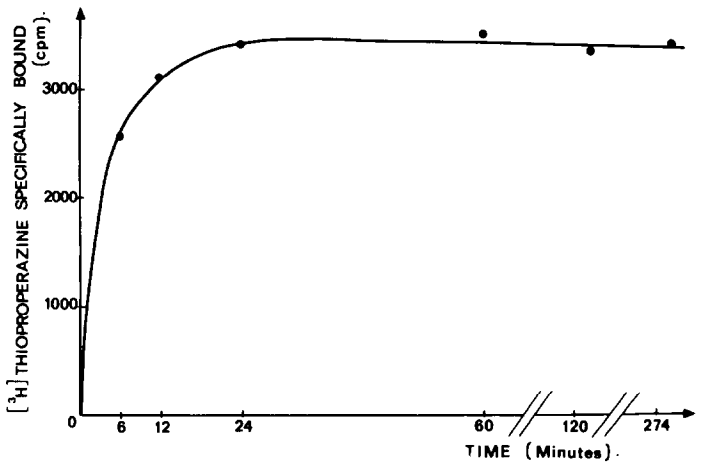

Fig. 4. Time course of [^{3}H]thioproperazine binding on microsomal striatal fraction.

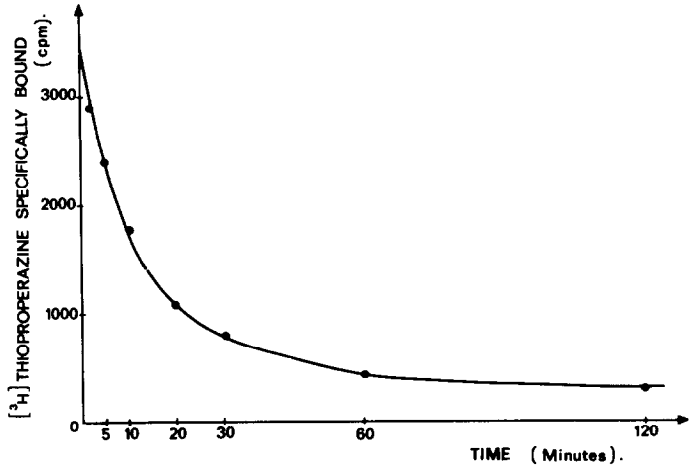

Fig. 5. Dissociation of [^{3}H]thioproperazine bound from a microsomal striatal fraction in the presence of an excess of cold thioproperazine. Each point is the mean of three determinations.

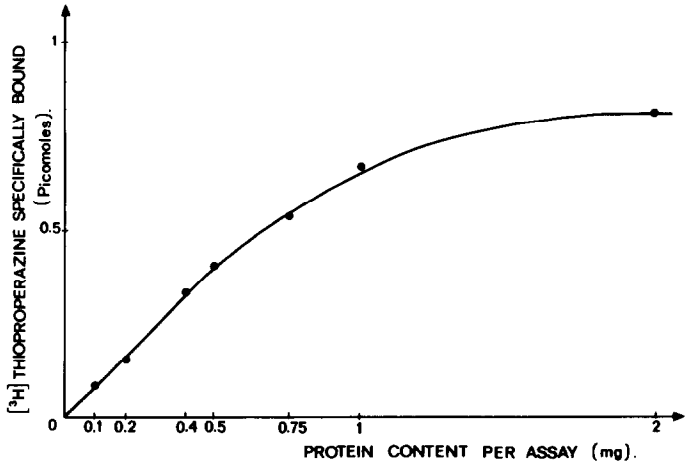

Fig. 6. Specific thioproperazine binding with increasing protein concentration using a LP fraction from rat striatum (ligand concentration = 4 nM). Each point is the mean of three determinations.

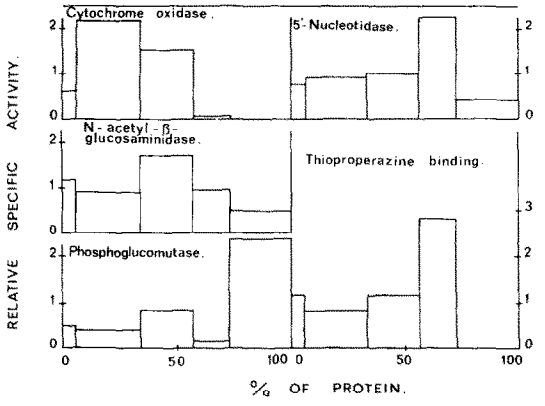

Fig. 7. Distribution pattern of enzymes and [^{3}H]thioproperazine binding in subcellular fractions obtained by differential centrifugation from rat striatum. Homogenate was divided into five fractions, N, M, L, P and S. These are represented by blocks ordered according to the same sequence on the abcissa where they span a length proportional to their protein content. The ordinate (heights of the blocks) give the relative specific activity (percentage of activity over percentage of protein) of the fractions. Percentages relate to the sum of the N, M, L, P and S fractions. Averaged recoveries from the homogenates ranged between 87 and 115 percent.

Table 1. Inhibition of [^{3}H]thioproperazine binding in rat striatum*

Compound	IC_{50} (nM)
Thioproperazine	0.87 ± 0.16
Pipotiazine	1.08 ± 0.17
Prochlorperazine	27 ± 2.35
Propericiazine	40 ± 18.2
Chlorpromazine	49.7 ± 7.82
Levomepromazine	49 ± 6.78
Thioridazine	109 ± 18.5
Metopimazine	3.31 ± 0.95
Spiperone	0.93 ± 0.24
Pimozide	3.24 ± 0.57
Haloperidol	9.28 ± 1.69
Pipamperone	295 ± 33
Domperidone	2.05 ± 0.15
Metoclopramide	229 ± 32
Sulpiride	284 ± 25
Bromocryptine	17 ± 1.9
Apomorphine	92 ± 9.25
Dopamine	1596 ± 200

* Compounds with IC_{50} values higher than 10,000 nM: atropine, serotonin, clonidine, phentolamine. For determination of IC_{50} value, each drug was tested at six concentrations. Results are the mean ± S.E.M. of at least three different experiments.

is 0.14×10^9 M^{-1} min^{-1}. The ratio of these rate constants yields a K_d of 0.2 nM, a value very similar to that found from the saturation isotherm.

Effect of protein concentration on specific [^{3}H]*thioproperazine binding.* Using a LP striatal fraction, specific thioproperazine binding is linear with protein content up to 0.5 mg of protein in the test (Fig. 6).

Regional and subcellular distribution of [^{3}H]*thioproperazine binding.* In addition to the striatum we studied the binding of [^{3}H]thioproperazine in other rat brain areas. We did not find a specific binding of thioproperazine in frontal cortex and hippocampus. However, in nucleus accumbens, an area rich in dopamine terminals, thioproperazine binds specifically. On the LP fraction of nucleus accumbens, the K_d measured by Scatchard analysis is 0.38 nM and the number of binding sites is 0.29 pmoles/mg protein. On a similar LP fraction from striatum, the K_d is 0.30 nM and the number of sites 0.9 pmoles/mg protein.

The subcellular distribution of [^{3}H]thioproperazine binding was studied in rat striatum. Figure 7 shows the subcellular distribution of various marker enzymes and that of binding sites. The mitochrondria marker enzyme, cytochrome oxidase, is found mainly in the M fraction, the lysosomal enzyme *N*-acetyl-*β*-glucosaminidase displays its highest relative specific activity in the L fraction. The maximum increase in binding sites is found in the P fraction where the plasma membrane marker enzyme 5′-nucleotidase shows its highest relative specific activity.

Inhibition studies of [^{3}H]*thioproperazine binding in the striatum.* Inhibition studies were performed on LP fractions of rat striata using a ligand concentration of 0.4 nM. For these studies, as for those presented before, we have used unlabelled thioproperazine to determine non-specific binding. This was allowed because, as shown in Fig. 8, the maximum displacement obtained with haloperidol, a butyrophenone neuroleptic, or apomorphine, a dopamine agonist, is equal to that observed with thioproperazine. Table 1 gives the results obtained with different compounds. We can observe that all neuroleptic compounds belonging to different chemical families are capable of displacing thioproperazine from its binding sites. The most potent drugs are spiperone, thioproperazine and pipotiazine. The sedative neuroleptics levomepromazine, chlorpromazine, thioridazine and pipamperone are weaker

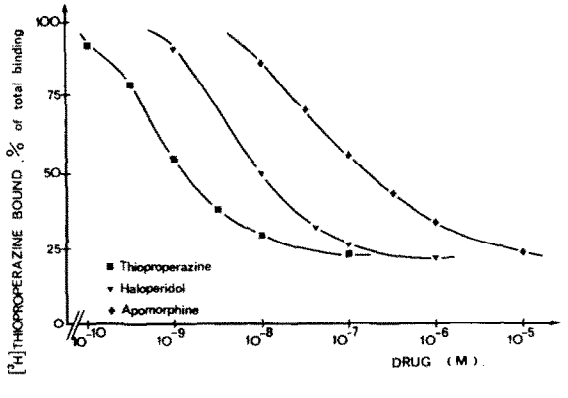

Fig. 8. Maximum displacement of [^{3}H]thioproperazine from a LP fraction of rat striatum by two dopamine antagonists (thioproperazine and haloperidol) and a dopamine agonist (apomorphine).

inhibitors. Sulpiride, a neuroleptic of the benzamide family, is also a weak inhibitor. Metopimazine, domperidone and metoclopramide, which are chemically related to different neuroleptic families but which are considered as devoid of neuroleptic activities, present clear affinity for the thioproperazine binding sites, the two first compounds being strong inhibitors. Dopamine and the two dopamine agonists, apomorphine and bromocryptine, present affinity for thioproperazine binding sites. Atropine, serotonin, clonidine and phentolamine have IC_{50} values higher than 10 μM.

As shown in Fig. 9, the (+) isomer, which is the neuroleptic active form of butaclamol, is about 1000 times more active than the (−) isomer.

DISCUSSION

From the experiments reported above, it can be concluded that [^{3}H]thioproperazine, a phenothiazine neuroleptic, is an appropriate ligand to label neuroleptic receptors in the rat and that the labelled sites are similar to those identified using some butyrophenones as ligands.

In rat striatum, [^{3}H]thioproperazine binding was found to be saturable and this saturable binding represents 80–90 per cent of the total binding, a percentage similar to that found with spiperone [5] and domperidone [8] but higher than that found with haloperidol [5]. Thus, the low aspecific binding of [^{3}H]thioproperazine must be underlined.

Data from Scatchard analysis reveal that thioproperazine binds, in the striatum, to one population of sites with a high affinity, the K_d value being 0.3 ± 0.03 nM. This K_d value is lower than that found for haloperidol and domperidone [5, 8]. On the other hand, the K_d value of thioproperazine is higher than that of spiperone; however, in the striatum and in other structures, spiperone probably labels a heterogeneous population of receptors [5, 16, 17].

The number of binding sites found in our experiments is 1.47 ± 0.11 pmoles/mg protein, a value very similar to those found by Leysen *et al.* [5] with spiperone (1.7 ± 0.1 pmoles/mg protein) and haloperidol (1.1 ± 0.1 pmoles/mg protein) using the microsomal fraction (P) as material receptor. These values are higher than those found by Burt *et al.* [3] for haloperidol; however, in this case, the study was performed on a particulate fraction.

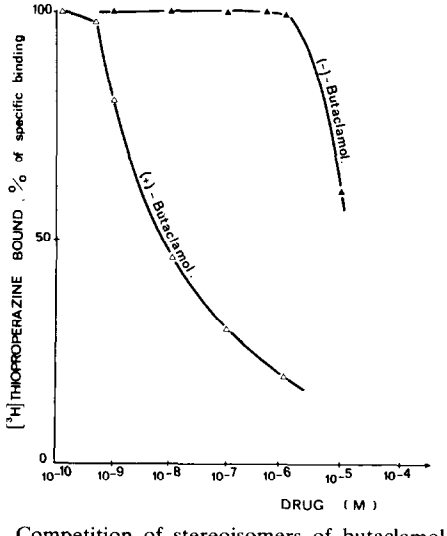

Fig. 9. Competition of stereoisomers of butaclamol for [^{3}H]thioproperazine binding site.

This specific high affinity thioproperazine binding has been observed in two dopaminergic areas, the striatum and the nucleus accumbens. Unexpectedly, specific binding has not been observed in the frontal cortex, although this region contains dopaminergic terminals and thioproperazine is capable of stimulating dopamine synthesis in this region (but using higher doses than those necessary to observe effects in striatum or nucleus accumbens [12]). Spiperone has been reported to bind in the frontal cortex, but in this region the neuroleptic receptor sites labelled by this drug could be serotonergic [16].

Subcellular distribution of [^{3}H]thioproperazine binding reveals that the maximum binding is observed in the microsomal fraction (P) which contains most of the plasma membrane marker 5′-nucleotidase. Similar distributions of spiperone and haloperidol binding have been found by Laduron *et al.* [18] using the same fractionation procedure.

All these results indicate that thioproperazine, a phenothiazine derivative, can be used as a ligand in binding studies and that the binding sites labelled by this phenothiazine are probably of similar nature to those revealed using haloperidol or spiperone. Furthermore, when different neuroleptics are tested for their inhibition against [^{3}H]thioproperazine binding in the rat striatum, all the neuroleptic drugs, even the butyrophenone derivatives, are capable of displacing thioproperazine from its binding sites. Moreover, there is a good correlation between the IC_{50} values against thioproperazine and the pIC_{50} values found by Leysen *et al.* [5] against haloperidol (correlation coefficient = 0.81) and spiperone (correlation coefficient = 0.90). Finally, dopamine and the two dopamine agonists, apomorphine and bromocryptine, also inhibit the thioproperazine binding. The two agonists are more potent inhibitors than the neurotransmitter; this has been observed for apomorphine using haloperidol [3], spiperone [5] or domperidone [8] as ligands.

Metopimazine, a non-neuroleptic phenothiazine, shows a high affinity for [^{3}H]thioproperazine binding which confirms the finding of Leysen *et al.* [5]. However, the results of the companion paper [10] show that *in vivo*, in the rat, metopimazine is unable to reach the thioproperazine binding sites, which could explain the lack of neuroleptic activity of this compound.

In conclusion, the phenothiazine derivative, thioproperazine, is a valuable ligand for labelling neuroleptic receptors in the rat. We have found only one binding site of high affinity; the nonspecific binding is low and similar to that of spiperone. The subcellular distribution of binding sites is similar to that of haloperidol and spiperone. Thioproperazine binds specifically in dopaminergic areas. All the neuroleptics tested are capable of displacing thioproperazine from its binding sites and their potencies against thioproperazine are similar to their potencies against a butyrophenone ligand. The agonist of

dopamine receptors apomorphine also inhibits thioproperazine binding. All these facts indicate similarity in the binding sites of phenothiazine and butyrophenone neuroleptics. As for haloperidol, the binding of thioproperazine is probably associated with dopamine receptors.

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REFERENCES

1. D. R. Burt, S. J. Enna, I. Creese and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4655 (1975).
2. P. Seeman, T. Lee, M. Chau-Wong, J. Tedesco and K. Wong, *Proc. natn Acad. Sci. U.S.A.* **73**, 4354 (1976).
3. D. R. Burt, I. Creese and S. H. Snyder, *Molec. Pharmac.* **12**, 800 (1976).
4. M. Titeler, J. L. Tedesco and P. Seeman, *Life Sci.* **23**, 587 (1978).
5. J. E. Leysen, W. Gommeren and P. M. Laduron, *Biochem. Pharmac.* **27**, 307 (1978).
6. D. Hauser and A. M. Closse, *Life Sci.* **23**, 557 (1978).
7. J. Hyttel, *Life Sci.* **23**, 551 (1978).
8. M. P. Martres, M. Baudry and J. C. Schwartz, *Life Sci.* **23**, 1781 (1978).
9. J. Delay, P. Deniker and J. M. Harl, *Ann. Med.-Psychol.* **110**, 112 (1952).
10. A. Boireau, J. C. Blanchard, C. Garret and L. Julou, *Biochem. Pharmac.* **29**, 000 (1980).
11. J. Delay and P. Deniker in *Méthodes Chimiothérapiques en Psychiatrie. Les Nouveaux Médicaments Psychotropes*, p. 165. Masson, Paris (1961).
12. L. Julou, B. Scatton and J. Glowinski, in *Advances in Biochemical Psychopharmacology* (Eds. E. Costa and G. L. Gessa), p. 617. Raven Press, New York (1977).
13. P. Tulkens, H. Beaufay and A. Trouet, *J. Cell Biol.* **63**, 383 (1974).
14. W. J. Ray and G. A. Roscelli, *J. biol. Chem.* **239**, 1228 (1964).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. J. E. Leysen, C. J. E. Niemegeers, J. P. Tollenaere and P. M. Laduron, *Nature, Lond.* **272**, 168 (1978).
17. N. W. Pedigo, T. D. Reisine, J. Z. Fields and H. I. Yamamura, *Eur. J. Pharmac.* **50**, 451 (1978).
18. P. M. Laduron, P. F. M. Janssen and J. E. Leysen, *Biochem. Pharmac.* **27**, 323 (1978).