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IRREVERSIBLE BLOCKADE OF CENTRAL 5-HT BINDING SITES BY 8-METHOXY-2′-CHLORO-PAT

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Abstract—We have synthesized 8-methoxy-2-(*N*-2′-chloropropyl, *N*-propyl) aminotetralin (8-methoxy-2′-chloro-PAT), an alkylating agent derived from the potent 5-HT agonist, 8-hydroxy-2-(*N*,*N*-dipropyl)-aminotetralin (PAT). As expected for an irreversible ligand, the blockade of ^{3}H-PAT or ^{3}H-5-HT binding to post-synaptic 5-HT$_1$ (A and B) sites in rat hippocampal membranes pretreated with 8-methoxy-2′-chloro-PAT could not be prevented by extensive washing of membranes. Prior occupancy of 5-HT$_1$ sites by 5-HT or PAT prevented any subsequent irreversible blockade by the alkylating agent. Similar irreversible blockade by 8-methoxy-2′-chloro-PAT was found on ^{3}H-PAT binding to striatal membranes suggesting that presynaptic 5-HT binding sites (see Gozlan *et al.*, *Nature, Lond.* **305**, 140, 1983) were sensitive also to the alkylating agent. In contrast, the modifying agent *N*-ethylmaleimide (NEM) reduced markedly ^{3}H-PAT binding to postsynaptic hippocampal 5-HT$_1$ sites, but did not alter ^{3}H-PAT binding to striatal presynaptic 5-HT sites. Although 8-methoxy-2′-chloro-PAT bound irreversibly to different classes of 5-HT binding sites (5-HT$_{1A}$, 5-HT$_{1B}$, presynaptic sites), it can be considered a selective alkylating agent, since it exerted no action on ^{3}H-spiperone binding to 5-HT$_2$ sites, ^{3}H-muscimol binding to GABA sites, or ^{3}H-flunitrazepam binding to benzodiazepine sites.

The present knowledge of the biochemical properties of central 5-HT‡ receptors is derived from two distinct experimental approaches: (1) the measurement of 5-HT-sensitive adenylate cyclase; and (2) the identification of specific binding sites for 5-HT-related ligands in brain membranes [1]. This second approach has been particularly fruitful during the last five years, mainly because of the availability of several ligands with high specific radioactivity [2]. However, these ligands are exclusively reversible and cannot be used for studying crucial problems such as the purification of specific binding sites or the turnover of these sites *in vivo*. Numerous examples in the literature have illustrated that such problems require irreversible ligands in order to be studied and possibly solved (see for instance [3, 4]).

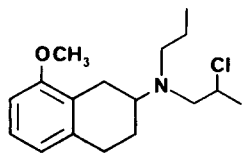
In the case of 5-HT receptors, only few attempts have been made to develop irreversible ligands. Cheng and Shih [5, 6] synthesized a photosensitive arylazide derivative of 5-HT (nitro-aryl-azidophenyl-5-HT or NAP-5-HT), which acts as an irreversible photoaffinity probe labelling some 5-HT-binding proteins in the rat brain. However, NAP-5-HT exhibits only μM affinity for 5-HT receptors (particularly 5-HT$_1$ sites) and its non-specific binding to non-5-HT related sites is relatively high, making the specific binding of this compound to 5-HT receptors difficult to quantify [5, 6]. More recently, Walker *et al.* [7] demonstrated that ^{3}H-LSD could be used for the irreversible photoaffinity labelling of some membrane proteins in liver fluke. However, LSD interacts not only with 5-HT receptors but also with dopamine and β-adrenergic receptors in brain (see [8]), therefore its use for selective studies of central 5-HT receptors is questionable.

Recently, Arvidsson *et al.* [9] synthesized a new tetralin derivative, 8-hydroxy-2-(*N*,*N*-dipropyl) aminotetralin (PAT), which was demonstrated to be a very potent and specific 5-HT agonist in the rat brain [10]. Furthermore, its tritiated derivative is a useful ligand for exploring both postsynaptic [11–13] and presynaptic [11, 12] 5-HT sites. This led us to choose the chemical structure of PAT as a model for the synthesis of some irreversible ligands of 5-HT receptors. In this paper, we describe the synthesis of one of these compounds, 8-methoxy-2-(*N*-2′-chloropropyl, *N*-propyl) aminotetralin (8-methoxy-2′-chloro-PAT). The specificity of this compound was assessed by examining its possible action not only on 5-HT sites (5-HT$_1$ A and B subsites, see ref. 14; 5-HT$_2$ sites, see ref. 15; presynaptic 5-HT sites, see ref. 11), but also on GABA and benzodiazepine receptors. Furthermore, the irreversible blockade of 5-HT binding sites by 8-methoxy-2′-chloro-PAT was compared to that already described following membrane exposure to the irreversible SH blocking agent, *N*-ethyl maleimide (see [16, 17]).

‡ Abbreviations used: PAT, 8-hydroxy-2-(*N*,*N*-dipropyl)aminotetralin; 5-HT, serotonin; NEM, *N*-ethylmaleimide; 8-methoxy-2′-chloro-PAT, 8-methoxy-2-(*N*-2′-chloropropyl, *N*-propyl) aminotetralin; GTP, guanosine tri-phosphate; GABA, γ-aminobutyric acid.

MATERIALS AND METHODS

Synthesis of 8-methoxy-2′-chloro-PAT. 1,7-Dihydroxynaphthalene was methylated using dimethyl-



8-methoxy-2'-chloro-PAT

sulphate [18] and reduced by Na in butanol to give 8-methoxytetralone [19]. Reductive amination with propylamine under an atmosphere of hydrogen, in the presence of palladium on carbon (10%) gave 8-methoxy-2-*N*-propylaminotetralin [20]. This compound (236 mg; 1.1 mmole) was alkylated by monobromoacetone (5 equiv.) in the presence of triethylamine (1.3 equiv.) in dimethylformamide (5 ml) for 7 hr at 70°. The solvent was evaporated and the residue was extracted with dichloromethane, washed with 0.3 N NaOH and water, and purified on a Silicagel column (eluent; ethylacetate/hexane, 2/1) to give 212 mg of 8-methoxy-2-(*N*-2-oxopropyl, *N*-propyl)-aminotetralin (yield = 70%). NMR ($CDCl_3$): 7.2 to 6.6 (m, 3H, aromatic); 3.8 (s, 3*H*, —O—C$\underline{H}_3$); 3.25 (*s*, 2*H*, —*N*—C$\underline{H}_2$—CO—); 2.2 (*s*, 3*H*, —CO—C$\underline{H}_3$); 0.9 (*t*, 3*H*, —CH_2—CH_2—C$\underline{H}_3$). This compound (212 mg, 0.77 mmole) was then reduced with lithium aluminium hydride (60 mg) in dry tetrahydrofuran (3 hr at 25°). The excess of reagent was destroyed with an aqueous solution of tetrahydrofuran (10%) and the reaction mixture was filtered. Following evaporation of the remaining solvent, the crude product was dissolved in ethylacetate, washed with water, and purified on a Silicagel column (eluent: chloroform/methanol, 10/1) to give 200 mg of 8-methoxy-2-(*N*-2'-hydroxypropyl,*N*-propyl)-aminotetralin (yield = 93%). NMR ($CDCl_3$): 7.2 to 6.6 (*m*, 3*H*, aromatic); 3.8 (*s*, 3*H*, —O—C$\underline{H}_3$); 1.1 (*d*, 3*H*, —CHOH—C$\underline{H}_3$); 0.9 (*t*, 3*H*, —CH_2—CH_2—C$\underline{H}_3$).Treatment of this compound (186 mg, 1.1 mmole) with a large excess of thionyl chloride (250 µl) in refluxing benzene gave 8-methoxy-2'-chloro-PAT hydrochloride. After neutralization of the reaction mixture with 0.3 N NaOH, 8-methoxy-2'-chloro-PAT was extracted with ether, purified on a Silicagel column (eluant: ethylacetate/hexane, 1/10) and finally treated with HCl in dry ether to form the hydrochloride (130 mg, yield = 60%). Purity of 8-methoxy-2'-chloro-PAT hydrochloride (a mixture of diastereoisomers) was checked by thin layer chromatography, high performance liquid chromatography (HPLC) and ^1H NMR spectrometry 100 MHZ ($CDCl_3$): 7.2 to 6.6 (*m*, 3*H*, aromatic); 4.1 to 3.8 (*m*, 1*H*, —C$\underline{H}$Cl—); 3.8 (*s*, 3*H*, —OC$\underline{H}_3$); 1.51 and 1.53 (*d*, 3*H*, —CHCl—C$\underline{H}_3$); 0.9 (*t*, 3*H*, —CH_2—CH_2—C$\underline{H}_3$).

Solvolysis of 8-methoxy-2'-chloro-PAT. The reaction was started by mixing 1.5 ml of a 2 mM solution of 8-methoxy-2'-chloro-PAT hydrochloride in ethanol with 1.5 ml of one of the following buffers: 0.1 M KH_2PO_4/K_2HPO_4, 0.1 M CH_3COONa, 0.1 M Tris–HCl, all adjusted to pH 7.4 at 37°. Samples were incubated at 37° and 0.1 ml aliquots were withdrawn at specific time intervals. Each aliquot was mixed with 0.1 ml 1 N H_2SO_4 at 0° to stop the reaction and its content was analysed immediately by HPLC using a C18 µBondapak column (mobile phase: 0.03 M triethylammonium phosphate, 30% acetonitrile, pH 3.0). Detection was monitored at 273 nm.

Preparation of membranes. Male Sprague–Dawley rats (200–250 g; Charles River) were killed by decapitation and the brain was rapidly removed and dissected in the cold (4°). Tissues were homogenized in 20 vol. (v/w) of ice-cold 0.05 M Tris–HCl buffer, pH 7.4, using a Polytron PT 10 OD disrupter. The homogenate was centrifuged at 40,000 *g* for 20 min and the resulting pellet was washed three times with 20 vol. of the same buffer. Following preincubation at 37° for 10 min [21], the membranes were collected by centrifugation, washed three times with 20 vol. of 0.05 M KH_2PO_4/K_2HPO_4, pH 7.4, and finally suspended in 10 vol. of this buffer.

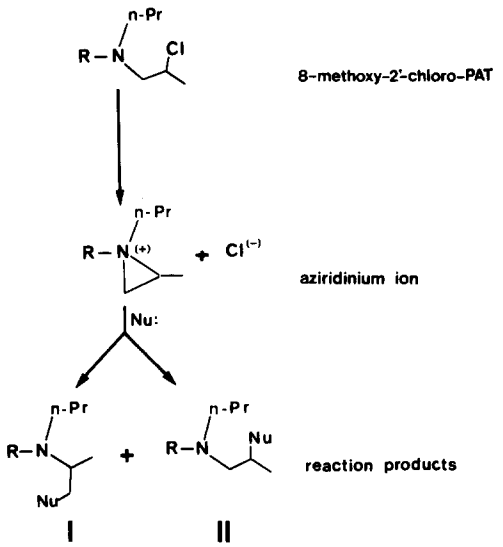
Treatment with 8-methoxy-2'-chloro-PAT. Membrane suspensions were diluted with 0.05 M potassium phosphate buffer containing 8-methoxy-2'-chloro-PAT (final conditions: 1 mg membrane prot./ml; 0–100 µM 8-methoxy-2'-chloro-PAT) and incubated for various times (usually 30 min) at 37°. This treatment was stopped by a fivefold dilution with 0.05 M Tris–HCl, pH 7.4, followed by centrifugation. The resulting pellets were washed six times in Tris–HCl buffer by resuspension and centrifugation (40,000 *g*, 10 min, 4°). In protection studies, when unlabelled PAT or 5-HT (5 µM) was added 5 min before 8-methoxy-2'-chloro-PAT, this washing procedure was found to totally eliminate these compounds from the membranes (data not shown).

NEM treatment. Membranes were washed twice with 20 vol. of 0.05 M Tris–HCl, pH 7.4, and then resuspended in the same buffer containing NEM (final conditions: 1 mg membrane prot./ml; 1 mM NEM). Following incubation for 10 min at 37°, samples were diluted fivefold with ice-cold Tris buffer and centrifuged (40,000 *g*, 10 min, 4°). The resulting pellets were washed as described for 8-methoxy-2'-chloro-PAT treatment.

Binding assays. Aliquots (0.2 ml) of membrane preparations (equivalent to 0.6–1.0 mg prot.) were added to 1.8 ml of 0.05 M Tris–HCl, pH 7.4, and incubated with one of the following ligands: ^3H-PAT (105 Ci/mmole, CEA Saclay, France), ^3H-5-HT (12.5 Ci/mmole, Amersham Int. plc, U.K.), ^3H-spiperone (21 Ci/mmole, Amersham Int. plc, U.K.), ^3H-muscimol (19 Ci/mmole, Amersham Int. plc, U.K.), or ^3H-flunitrazepam (86 Ci/mmole, Amersham Int. plc, U.K.), according to published procedures for the specific labelling of 5-HT_1 [11, 21], 5-HT_2, GABA and benzodiazepine [22] binding sites respectively. Specific binding was defined as the difference between total radioactivity bound to membranes minus that persisting in the presence of a large excess of an appropriate unlabelled ligand (10 µM 5-HT for ^3H-5-HT and ^3H-PAT binding; 1 µM cinanserin for ^3H-spiperone binding; 0.1 mM GABA for ^3H-muscimol binding; 10 µM diazepam for ^3H-flunitrazepam binding). For each condition, binding assays were performed in triplicate.

Protein was measured using the Folin phenol procedure [23] with bovine serum albumin as the standard.

Statistical calculations were made according to



Scheme 1. Probable mechanism of the irreversible blockade of 5-HT binding sites by 8-methoxy-2'-chloro-PAT. 8-methoxy-2'-chloro-PAT first gives an aziridinium ion which then reacts with various nucleophilic groups present in the incubation medium or at the 5-HT binding sites. Two types of nucleophilic substitution products (I, II) can be formed (see ref. 28). Pr: propyl group; Nu: Nucleophilic group.

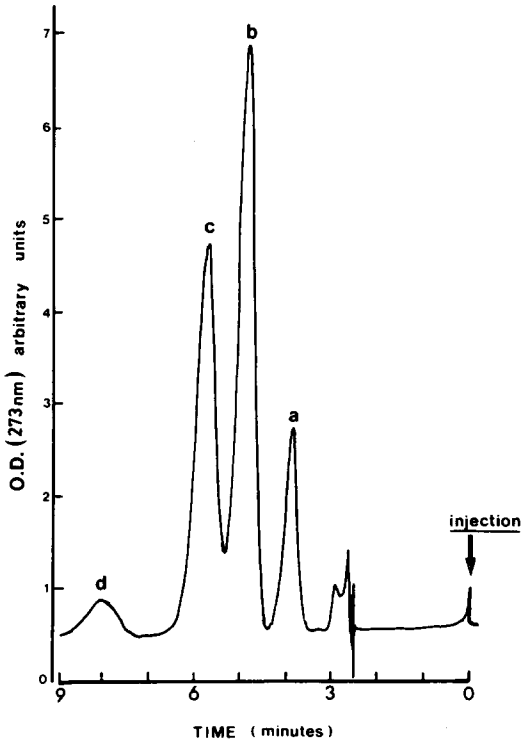


Fig. 1. HPLC profile of the products formed after a 3 min incubation of 8-methoxy-2'-chloro-PAT at 37° in a mixture of 0.05 M potassium phosphate buffer (pH 7.5) and ethanol (1:1 by vol.) HPLC was run on a C18 μ Bondapak column using an isocratic mode (mobile phase: 0.03 M triethylammonium phosphate: acetonitrile, 7:3 by vol., pH 3.0; 1 ml/min). Eluted compounds were detected by measuring the optical density at 273 nm. For this particular experiment, 100 μl of the incubated 8-methoxy-2'-chloro-PAT solution (1 mM, 3 min, 37°) were injected. 0 = injection time. Peaks a and d: solvolysis products of the aziridinium ion; peak b: aziridinium ion; peak c: 8-methoxy-2'-chloro-PAT.

Snedecor and Cochran [24] with $P = 0.05$ as the limit for significant differences (unpaired two-tailed Student's *t*-test).

RESULTS

Reactivity of 8-methoxy-2'-chloro-PAT

8-Methoxy-2'-chloro-PAT is a 2-chloroamine derivative, like phenoxybenzamine [25, 26], *N*-(2-chloroethyl)norapomorphine [27] and other mustard compounds [28], and thus should react through an intermediate aziridinium ion [28], as illustrated in Scheme 1. Indeed, this was observed by monitoring the nucleophilic reaction of 8-methoxy-2'-chloro-PAT in various buffers by HPLC. A typical elution profile of the products formed in potassium phosphate buffer is shown in Fig. 1. In addition to 8-methoxy-2'-chloro-PAT (peak c), three other major peaks were found. Peak b probably corresponded to the aziridinium ion, since it was found in all buffers used (phosphate, acetate or Tris–HCl). Furthermore, as expected for an intermediate product (see [28]), its concentration increased during an initial phase and then decreased (Fig. 2). The decaying phase was due to the nucleophilic reaction of the aziridinium ion with water and phosphate. Peak d (Figs. 1 and 2) probably resulted from water opening of the aziridinium ion, since it occurred in each buffer examined. Peak a (Figs. 1 and 2) was observed only in potassium phosphate buffer and could correspond to a solvolysis product due to phosphate opening of the aziridinium ion.

Kinetic analysis of the reaction in potassium phosphate buffer (Fig. 2) indicated that 8-methoxy-2'-chloro-PAT and the corresponding aziridinium ion had a half life of 2 min and 12.5 min respectively. Based on the disappearance of the sum 8-methoxy-2'-chloro-PAT plus the aziridinium ion, the calculated half life of compounds potentially active as irreversible ligands of 5-HT binding sites approximated 10 min under those conditions.

Interaction of 8-methoxy-2'-chloro-PAT with 5-HT binding sites

Previous studies [11] have demonstrated that ^{3}H-PAT is a suitable ligand for labelling both postsynaptic 5-HT$_1$ sites in the hippocampus and presynaptic 5-HT sites in the striatum. This led us to select these two brain regions for investigating the possible action of 8-methoxy-2'-chloro-PAT on specific 5-HT binding sites. Addition of inframicromolar concentrations of 8-methoxy-2'-chloro-PAT to the incubating medium during ^{3}H-PAT binding assays resulted in a marked inhibition of ligand binding to hippocampal and striatal membranes (Fig. 3).

Analysis of the concentration-dependent inhi-

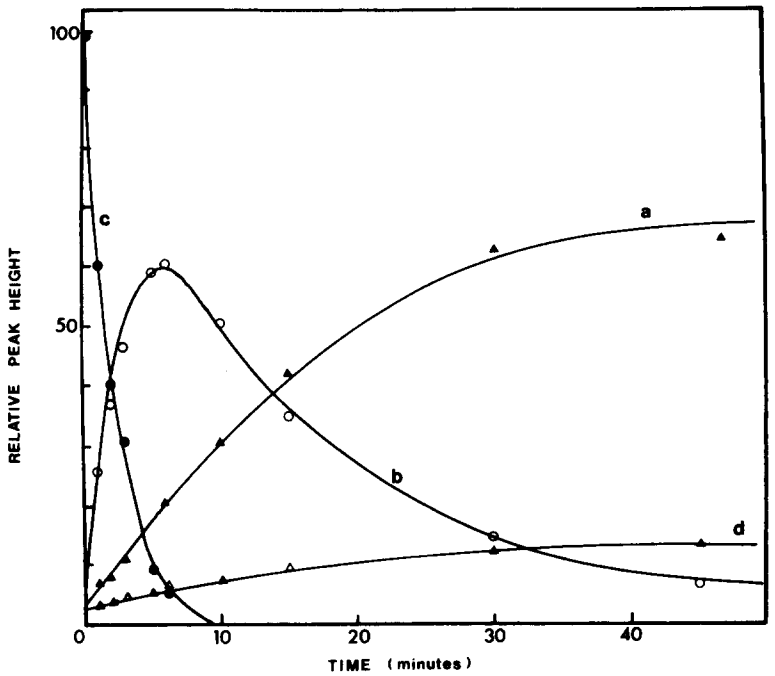


Fig. 2. Evolution of 8-methoxy-2'-chloro-PAT conversion during its incubation at 37° in a mixture of 0.05 M potassium phosphate buffer (pH 7.5) and ethanol (1:1 by vol.). An aliquot (100 μl) of the 8-methoxy-2'-chloro-PAT solution (1 mM) was taken at various times during the incubation, and treated for HPLC analysis (see Materials and Methods). Relative peak heights (8-methoxy-2'-chloro-PAT at 0 time = 100) are plotted as a function of incubation time. Symbols correspond to the peaks in Fig. 1 as follows: peak a (▲); peak b (○); peak c (●); peak d (△).

bition curve in the hippocampus (Fig. 3) estimated the IC_{50} value to be approximately 80 nM. However, the efficacy of 8-methoxy-2'-chloro-PAT was much less in the striatum, since the estimated IC_{50} value was around 1.5 μM for inhibition of ^{3}H-PAT binding in this brain region (Fig. 3). Obviously, such IC_{50} values were more apparent than real, taking into account the complexity of the reaction mixture during the 10 min incubation time (see Fig. 2).

Exposure of membranes to either 5 μM 5-HT or PAT followed by extensive washing did not alter ^{3}H-PAT binding capacity. However, preincubation of membranes with 8-methoxy-2'-chloro-PAT and subsequent washing did not allow complete recovery of ^{3}H-PAT binding capacity. We observed a 10% reduction in specific ^{3}H-PAT binding when hippocampal membranes were preincubated with 1 μM of the chloro-derivative for 10 min at 37°. This effect was time-dependent and reached a maximum after a 30 min incubation at 37° with 8-methoxy-2'-chloro-PAT (~55% inhibition with 1 μM 8-methoxy-2'-chloro-PAT). When the preincubation time was fixed at 30 min, 5-HT binding sites were blocked in a concentration-dependent manner by 8-methoxy-2'-chloro-PAT, and a complete blockade of ^{3}H-PAT binding sites was reached using 30 μM 8-methoxy-2'-chloro-PAT (Fig. 4). In contrast to that observed under equilibrium conditions (Fig. 3), no significant difference existed between the respective efficacies of 8-methoxy-2'-chloro-PAT as an irreversible blocker of ^{3}H-PAT binding sites in hippocampal and striatal membranes (Fig. 4).

Characteristics of 5-HT sites in membranes exposed to a submaximal concentration of 8-methoxy-2'-chloro-PAT; comparison with those after membrane exposure to NEM

Scatchard analysis (Fig. 5, Table 1) revealed that membrane exposure to 1 μM 8-methoxy-2'-chloro-PAT for 30 min resulted in a ~50% reduction of the number of specific ^{3}H-PAT binding sites in the hippocampus and striatum. This reduction reached ~75% using 10 μM of the alkylating agent (Table 1). However, the apparent affinity ($K_a = K_d^{-1}$) of ^{3}H-PAT binding sites which persisted after membrane treatment with 8-methoxy-2'-chloro-PAT was not significantly different from that found in control membranes (Table 1). These selective changes in B_{max} could be prevented by the prior addition of an excess of unlabelled PAT or 5-HT ensuring a complete occupancy of specific sites before incubation with 8-methoxy-2'-chloro-PAT (Fig. 5, Table 1). However, when this protection was achieved with 0.5 mM 5-HT, a significant increase in K_d values was found in both hippocampal and striatal membranes (Table 1).

Further experiments conducted with ^{3}H-5-HT instead of ^{3}H-PAT as the labelled ligand gave similar results: 8-methoxy-2'-chloro-PAT (1 μM, 30 min) induced a marked loss (−43%) of specific 5-HT_{1A} sites in hippocampal membranes, and occupancy of these sites with "cold" PAT or 5-HT insured a complete protection from subsequent blockade by the alkylating agent (data not shown).

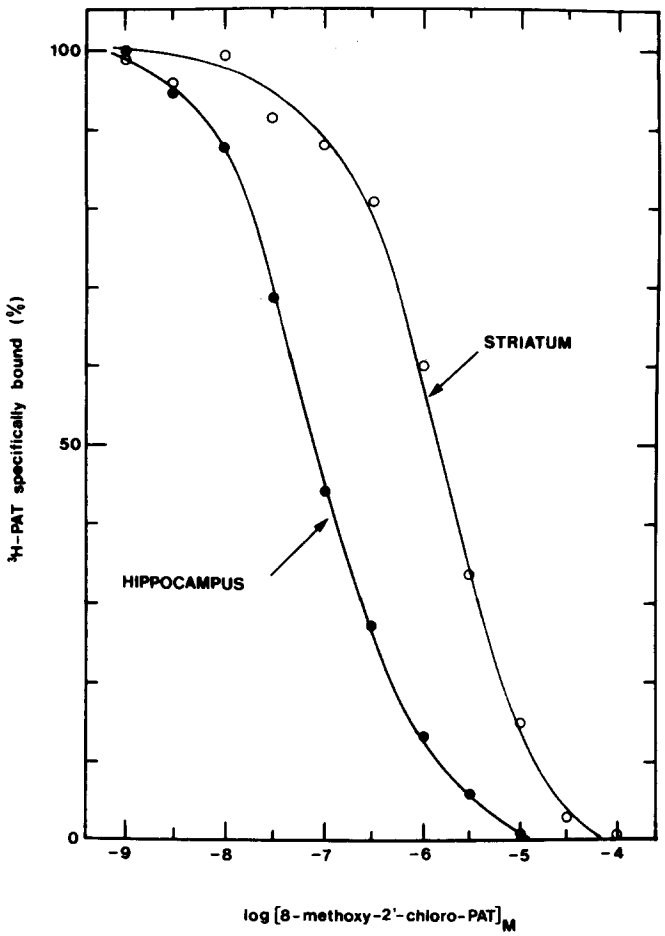


Fig. 3. Inhibition of ^{3}H-PAT binding to hippocampal and striatal membranes by 8-methoxy-2'-chloro-PAT. Membranes were incubated for 10 min at 37° in 0.05 M Tris–HCl, pH 7.4, containing 1 nM ^{3}H-PAT and various concentrations (1 nM–0.1 mM) of 8-methoxy-2'-chloro-PAT. Specific ^{3}H-PAT binding was defined as that prevented by co-incubation with 10 μM 5-HT. Each point (mean of triplicate determinations in three independent experiments) corresponds to specific ^{3}H-PAT binding expressed in percentage of that found in the absence of 8-methoxy-2'-chloro-PAT (100%). These values were respectively: 100% = 126. 4 fmoles/mg prot. with hippocampal membranes; 100% = 28.4 fmoles/mg prot. with striatal membranes.

Previous studies have indicated that NEM irreversibly blocks 5-HT binding sites in brain membranes [16, 17], thus experiments were then carried out in order to compare the inhibitory effects of NEM and 8-methoxy-2'-chloro-PAT on ^{3}H-PAT binding. As shown in Table 2, NEM treatment induced a marked reduction in ^{3}H-PAT specific binding to hippocampal (−74%) membranes, but not to striatal membranes. Such regional differences clearly differed from that observed following membrane treatment with 8-methoxy-2'-chloro-PAT (Fig. 4, Table 1) therefore suggesting a relative independency between the respective irreversible effects of NEM and 8-methoxy-2'-chloro-PAT on ^{3}H-PAT binding. This was confirmed in experiments which consisted of exposing membranes to both agents. When membranes were preincubated with 8-methoxy-2'-chloro-PAT followed, after washing, by a preincubation with NEM, the inhibitory effects of these two agents together were found to be partially additive (Table 2).

Distinction between irreversible blockade of ^{3}H-PAT binding sites by 8-methoxy-2'-chloro-PAT and NEM was assessed also by examining the modulatory effects of GTP and Mn^{2+} on hippocampal 5-HT_{1A} sites. In agreement with earlier reports [11, 17], we observed that GTP and Mn^{2+} induced opposite affinity changes of ^{3}H-PAT binding to untreated hippocampal membranes (Table 3). Similar modulations were seen on ^{3}H-PAT binding which persisted after 8-methoxy-2'-chloro-PAT treatment. In contrast, neither GTP nor Mn^{2+} significantly affected ^{3}H-PAT binding to hippocampal membranes previously exposed to NEM (Table 3).

Since ^{3}H-PAT is a selective ligand of the 5-HT_{1A} subsite at the postsynaptic level [10, 13, 29, 30], one could expect that its halogenated derivative 8-methoxy-2'-chloro-PAT affected preferentially this category of 5-HT sites. This led us to estimate the relative proportions of 5-HT_{1A} and 5-HT_{1B} subsites in hippocampal membranes before and after exposure to 1 μM 8-methoxy-2'-chloro-PAT. In agreement with previous findings [10, 14, 28], 5-HT_{1A} and 5-HT_{1B} subsites could be easily dis-

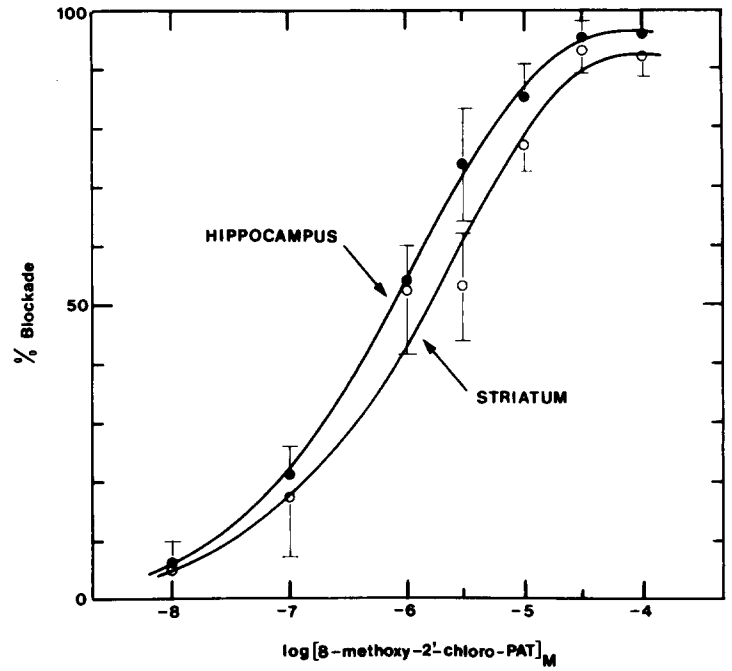


Fig. 4. Concentration-dependent irreversible blockade of ^{3}H-PAT binding to hippocampal and striatal membranes by 8-methoxy-2'-chloro-PAT. Membranes were preincubated for 30 min at 37° with various concentrations (10 nM–0.1 mM, abscissa) of 8-methoxy-2'-chloro-PAT and then washed as described in Materials and Methods. ^{3}H-PAT binding assays were carried out with 1 nM of the labelled ligand. Each point corresponds to the mean ± S.E.M. (4 separate experiments) of the reduction in specific ^{3}H-PAT binding resulting from preincubation with 8-methoxy-2'-chloro-PAT. Specific ^{3}H-PAT binding following preincubation in the absence of the alkylating agent and washing was equal to 108.5 ± 4.7 fmoles/mg prot. with hippocampal membranes, and 24.2 ± 2.0 fmoles/mg prot. with striatal membranes. Non specific binding (in the presence of 10 μM 5-HT) was unaffected by the precincubation with 10 nM–0.1 mM 8-methoxy-2'-chloro-PAT.

tinguished by their respective (high and low) affinities for spiperone (Fig. 6-I) or PAT (Fig. 6-II). In control membranes, the biphasic inhibition of ^{3}H-5-HT binding by increasing concentrations of spiperone (Fig. 6-I) or PAT (Fig. 6-II) allowed an estimate of 66–70% and 30–34% for the respective proportions of 5-HT_{1A} and 5-HT_{1B} subsites. Since similar proportions were found for the sites which persisted following membrane exposure to 8-methoxy-2'-chloro-PAT (Fig. 6-I, II), it could be inferred that this chloro-derivative equally reduced ^{3}H-5-HT-binding to both classes of subsites. In this respect, 8-methoxy-2'-chloro-PAT treatment resembled NEM treatment which affected to the same extent ^{3}H-5-HT binding to 5-HT_{1A} and 5-HT_{1B} subsites in the hippocampus (data not shown).

Effects of 8-methoxy-2'-chloro-PAT on other binding sites in brain membranes

Under conditions producing an irreversible blockade of postsynaptic 5-HT_1 sites (in the hippocampus) and of presynaptic sites (in the striatum), i.e. membrane incubation with 1–10 μM 8-methoxy-2'-chloro-PAT for 30 min at 37°, the specific binding of ^{3}H-spiperone to 5-HT_2 sites in membranes from the rat cerebral cortex remained unaltered (data not shown). Similarly, pretreatment of hippocampal membranes with 1–10 μM 8-methoxy-2'-chloro-PAT did not affect specific ^{3}H-flunitrazepam and ^{3}H-muscimol binding to benzodiazepine and GABA receptors respectively (not shown).

DISCUSSION

Several observations indicated that the chloro-derivative of PAT presently studied is in fact an alkylating agent producing a selective irreversible blockade of 5-HT binding sites in rat brain membranes:

(1) Extensive repeated washings of membranes preincubated with 8-methoxy-2'-chloro-PAT were not able to reverse its inhibitory action on ^{3}H-PAT or ^{3}H-5-HT binding.

(2) Neither the 5-HT_2 site labelled by ^{3}H-spiperone, nor benzodiazepine or GABA sites were affected by 8-methoxy-2'-chloro-PAT.

(3) The occupancy of 5-HT sites by an excess of PAT or 5-HT prevented the subsequent blockade of ^{3}H-5-HT or ^{3}H-PAT binding sites by 8-methoxy-2'-chloro-PAT. In the case of inhibition by 10 μM 8-methoxy-2'-chloro-PAT, complete protection (i.e. no significant reduction in the B_{max} of ^{3}H-PAT binding sites) achieved using 500 μM 5-HT was associated with a significant decrease in the apparent affinity of the specific sites for the labelled ligand. This effect probably resulted from the persistence of some 5-HT adsorbed to brain

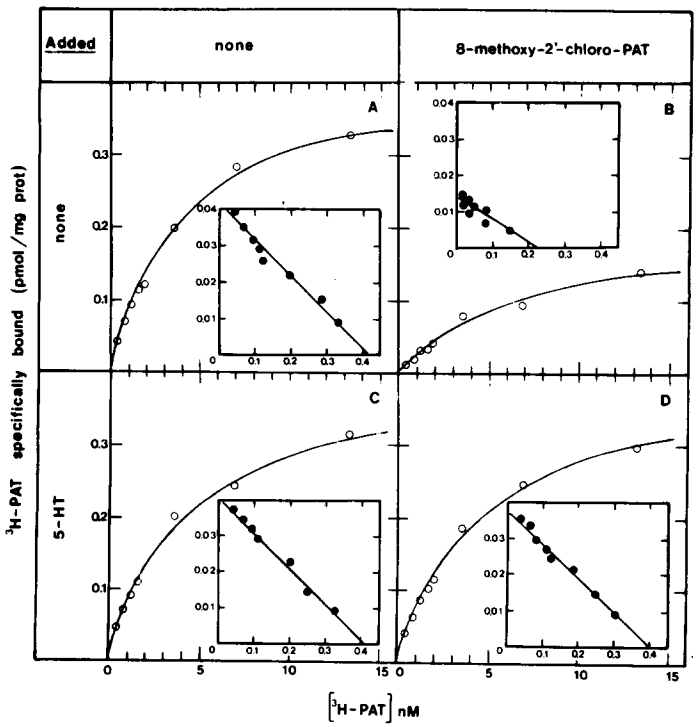


Fig. 5. Protection by 5-HT from irreversible blockade of ^{3}H-PAT binding sites in hippocampal membranes exposed to 8-methoxy-2′-chloro-PAT. Hippocampal membranes (1 mg prot./ml) were preincubated for 30 min at 37° with the appropriate ligands (A: none; B: 1 μM 8-methoxy-2′-chloro-PAT; C: 5 μM 5-HT; D: 5 μM 5-HT + 1 μM 8-methoxy-2′-chloro-PAT) in 0.05 M potassium phosphate buffer, pH 7.4, and washed six times as described in Materials and Methods. ^{3}H-PAT binding was determined using 0.3–13.2 nM of the labelled ligand. Each point is the mean of triplicate determinations. Insets: Linear regression analysis of Scatchard plots of the specific binding of ^{3}H-PAT; abscissa: ^{3}H-PAT specifically bound (B, in pmoles/mg prot.); ordinate: B/F, F being the amount (in pmoles) of free ^{3}H-PAT in samples.

Table 1. Protection of ^{3}H-PAT binding sites in hippocampal and striatal membranes from irreversible blockade by 8-methoxy-2′-chloro-PAT using unlabelled PAT or 5-HT

	Hippocampus		Striatum	
Treatment	K_d (nM)	B_{max} (pmoles/mg prot)	K_d (nM)	B_{max} (pmoles/mg prot)
None	2.7 ± 0.5	0.400 ± 0.021	12.4 ± 1.8	0.257 ± 0.019
8-Methoxy-2′-chloro-PAT				
1 μM	4.6 ± 1.3	0.208* ± 0.009	11.1 ± 2.2	0.122* ± 0.013
1 μM + 5 μM PAT	4.1 ± 1.2	0.426 ± 0.021	14.4 ± 1.8	0.233* ± 0.014
1 μM + 5 μM 5-HT	3.4 ± 0.7	0.425 ± 0.026	—	—
10 μM	5.2 ± 1.7	0.103* ± 0.015	10.8 ± 2.1	0.062* ± 0.013
10 μM + 500 μM 5-HT	12.5* ± 2.9	0.335 ± 0.034	19.6* ± 3.4	0.248 ± 0.024

Hippocampal or striatal membranes were preincubated for 30 min at 37° with 1 or 10 μM 8-methoxy-2′-chloro PAT in the presence or the absence of PAT (5 μM) or 5-HT (5–500 μM) as indicated in the left-hand column. After extensive washing (see Materials and Methods), membranes were used for binding assays with 0.3–15 nM ^{3}H-PAT. Linear regression analysis of Scatchard plots allowed the calculation of K_d (in nM) and B_{max} (in pmoles/mg prot) for each batch of membranes. Each value is the mean ± S.E.M. of determinations made in 3–6 independent experiments.

* $P < 0.05$ when compared to respective control ("no treatment") values.

Table 2. Comparison of the irreversible effects of 8-methoxy-2'-chloro-PAT and NEM on specific ^{3}H-PAT binding to hippocampal and striatal membranes

Treatment	^{3}H-PAT specifically bound (fmoles/mg prot)	
	Hippocampus	Striatum
None	130.5 ± 4.8	31.4 ± 1.3
8-methoxy-2'-chloro-PAT (1 μM)	61.3* ± 4.4 (−53%)	10.9* ± 1.0 (−65%)
NEM (1 mM)	33.7* ± 2.7 (−74%)	31.1 ± 1.7 (−1%)
8-methoxy-2'-chloro-PAT (1 μM) + NEM (1 mM)	10.3*† ± 1.6 (−92%)	11.1*† ± 0.9 (−65%)

Membranes were preincubated with 8-methoxy-2'-chloro-PAT (1 μM), NEM (1 mM) or both and then washed as described in Materials and Methods. Binding assays were performed in triplicate using 1.15 nM of ^{3}H-PAT. Each value is the mean ± S.E.M. of data obtained in 5 independent experiments. The per cent reduction due to various treatments is given in parentheses. Neither 8-methoxy-2'-chloro-PAT, nor NEM treatment affected the non-specific binding of ^{3}H-PAT (in the presence of 10 μM 5-HT) to hippocampal and striatal membranes.

* $P < 0.05$ when compared to respective control ("no treatment") values.

† $P < 0.05$ when compared to values obtained for membranes treated with NEM alone.

membranes even after repeated extensive washings. Starting with such a large concentration of 5-HT, an optimal washing procedure should have involved an incubation step at 37° in order to accelerate the release of 5-HT bound to membranes (see [21]); in the present case, this was not attempted in order to avoid any possible inactivation of the binding sites.

Experiments designed to define the appropriate conditions for reaching maximal irreversible blockade revealed that a 30 min incubation with 8-methoxy-2'-chloro-PAT were required although this compound was no longer present in the medium after the 10th min of incubation (its half-life was approximately 2 min). Such discrepancy clearly indicates that 8-methoxy-2'-chloro-PAT itself was not the true alkylating agent. After its binding to 5-HT sites, it was rapidly cyclized to the aziridinium ion, which then reacted with active groups to achieve the irreversible blockade of these sites (see Scheme 1). As expected for such an interpretation, irreversible blockade plateaued after 30 min of incubation when the aziridinium ion had almost completely disappeared from the incubation medium. Previous studies with other chloramines which block irreversibly monoamine receptors such as phenoxybenzamine [25, 26] and *N*-(2-chloroethyl) norapomorphine [27], have shown also that the aziridinium ions which are formed by cyclization of these molecules are in fact the true alkylating agents [28].

Like PAT [11, 12], 8-methoxy-2'-chloro-PAT was better recognized by specific sites in hippocampal than striatal membranes. This was illustrated by the results of experiments which consisted of incubating

Table 3. Effects of GTP or Mn^{2+} on the specific binding of ^{3}H-PAT to hippocampal membranes pretreated or not with 8-methoxy-2'-chloro-PAT or NEM

Addition	^{3}H-PAT specifically bound (fmoles/mg prot)		
	Control	8-Methoxy-2'-chloro-PAT	NEM
None	126.7 ± 9.0	53.3 ± 5.4	28.7 ± 2.7
GTP (0.1 mM)	40.7* ± 4.5 (−68%)	22.9* ± 2.9 (−57%)	24.0 ± 2.4 (−16%)
Mn^{2+} (1 mM)	190.6* ± 3.7 (+50%)	82.3* ± 9.3 (+54%)	26.6 ± 3.0 (−7%)

Hippocampal membranes were preincubated with 8-methoxy-2'-chloro-PAT (1 μM) or NEM (1 mM) and then washed as described in Materials and Methods. The specific binding of ^{3}H-PAT was measured with 1.10 nM of the labelled ligand in the absence (none) or the presence of 0.1 mM GTP or 1 mM $MnCl_2$. Each value is the mean ± S.E.M. of data obtained in 4 independent experiments. The per cent change due to GTP or Mn^{2+} is indicated in parentheses.

* $P < 0.05$ when compared to respective ^{3}H-PAT binding in the absence of GTP and Mn^{2+}.

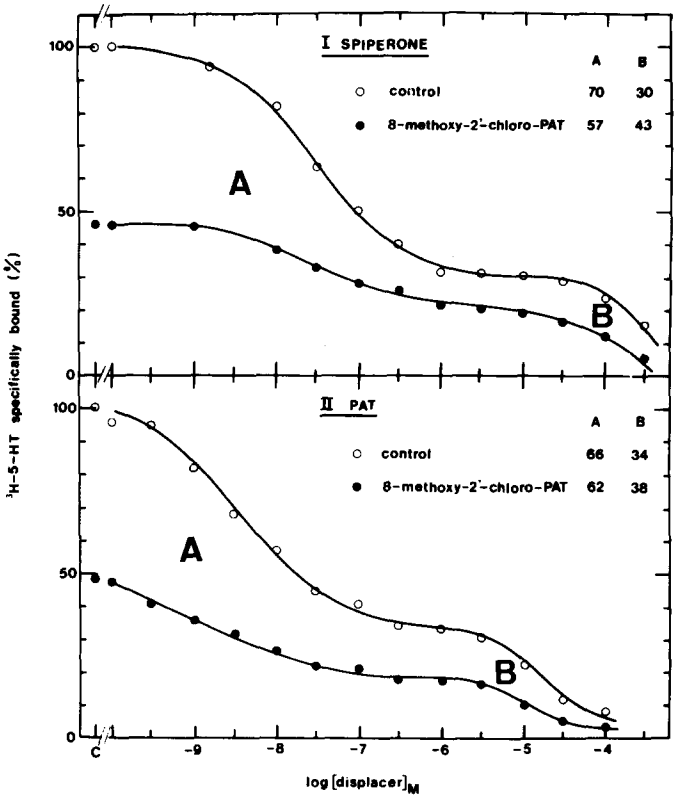


Fig. 6. Inhibition by spiperone (I) or PAT (II) of ^{3}H-5-HT binding to hippocampal membranes preincubated in the absence or presence of 8-methoxy-2'-chloro-PAT. Hippocampal membranes were preincubated for 30 min at 37° in the absence ("control") or presence of 1 μM 8-methoxy-2'-chloro-PAT and then washed as described in Materials and Methods. Specific ^{3}H-5-HT binding was measured with 1.3 nM of the labelled ligand in the presence of various concentrations (0.1 nM–0.3 mM) of spiperone (I) or PAT (II). Data are expressed in percentage of ^{3}H-5-HT bound to control membranes in the absence of spiperone or PAT (100% = 75.4 fmoles/mg prot.). Each point is the mean of triplicate determinations in two separate experiments. ○, control membranes (preincubated without 8-methoxy-2'-chloro-PAT); ●, membranes preincubated with 1 μM 8-methoxy-2'-chloro-PAT; A, proportion of ^{3}H-5-HT binding to 5-HT$_{1A}$ subsite, as a percentage of total specific binding; B, proportion of ^{3}H-5-HT binding to 5-HT$_{1B}$ subsite (100-A).

membranes for only 10 min in the presence of both ^{3}H-PAT and 8-methoxy-2'-chloro-PAT. Under such conditions, the inhibition of ^{3}H-PAT binding was almost completely reversible (by extensive washing) therefore allowing the calculation of apparent IC_{50} values of 8-methoxy-2'-chloro-PAT. These values, 80 nM in the hippocampus and 1.5 μM in the striatum, were obviously overestimates of the actual IC_{50} values since 8-methoxy-2'-chloro-PAT and the corresponding aziridinium ion disappeared with a half-life of about 10 min in the incubating medium.

Despite such differences under equilibrium conditions (10 min, 37°), 8-methoxy-2'-chloro-PAT was equipotent in the striatum and hippocampus for blocking irreversibly ^{3}H-PAT binding sites. This suggests that the limiting step in the sequence between the binding of 8-methoxy-2'-chloro-PAT to 5-HT sites and the resulting irreversible blockade was not solely dependent on the affinity of these sites for the chloro-derivative of PAT. Several hypotheses can be put forward to explain such differences between reversible and irreversible interactions of 8-methoxy-2'-chloro-PAT and ^{3}H-PAT binding sites in the hippocampus and striatum. Since the physico-chemical properties of the hippocampal and striatal ^{3}H-PAT binding sites are markedly different [31], it can be proposed that the striatal environment made the alkylation process by the aziridinium ion much easier than the hippocampal environment. Alternatively, possible occupancy of ^{3}H-PAT binding sites by the solvolysis products of the aziridinium ion might protect the hippocampal sites to a greater extent than the striatal sites from irreversible blockade by the alkylating agent. Further experiments (for instance the measurement of the respective affinities of striatal and hippocampal ^{3}H-PAT binding sites for these solvolysis products) are necessary to fully understand such differences in the mechanisms involved in the irreversible blockade of hippocampal and striatal ^{3}H-PAT binding sites by 8-methoxy-2'-chloro-PAT.

On the basis of subcellular distribution and lesion studies, Gozlan *et al.* [11] proposed that ^{3}H-PAT binding sites were postsynaptic in the hippocampus, and presynaptic in the striatum. Recent evidence has confirmed that the ^{3}H-PAT binding site in the hippocampus is, in fact, identical to the 5-HT$_{1A}$

subsite [10, 13]. However, some controversy [10, 32, 33] still persists concerning the possible correspondence of presynaptic ^{3}H-PAT binding sites found in the rat striatum to the presynaptic 5-HT autoreceptors controlling 5-HT release. In this respect, 8-methoxy-2′-chloro-PAT might well be a useful drug to solve the problem since the present study indicated that 8-methoxy-2′-chloro-PAT is a potent irreversible blocker of presynaptic striatal ^{3}H-PAT binding sites. Whether such blockade suppresses the negative feed-back control of 5-HT release mediated by presynaptic 5-HT autoreceptors is currently under investigation in our laboratory.

In contrast to 8-methoxy-2′-chloro-PAT which blocked equally postsynaptic 5-HT$_1$ (A and B) sites and presynaptic ^{3}H-PAT binding sites, the modifying agent NEM affected only the postsynaptic sites. Such findings further confirmed the differences in the physico-chemical environments of post- and pre-synaptic sites since they suggested that SH groups play a critical role in the ligand binding to postsynaptic sites only. Furthermore, such SH groups are involved in the modulatory effects of GTP and Mn^{2+} on postsynaptic 5-HT$_1$ sites [17] since exposure of hippocampal membranes to NEM made the persisting ^{3}H-PAT binding sites no longer sensitive to these agents.

In conclusion, 8-methoxy-2′-chloro-PAT appears to be a useful tool for studying 5-HT binding sites in the rat brain since it is a specific alkylating agent irreversibly inhibiting presynaptic ^{3}H-PAT binding sites in the striatum and postsynaptic 5-HT$_1$ A and B sites in the hippocampus. Previous studies have shown that low molecular weight chloramines, such as phenoxybenzamine, pass the blood brain barrier and bind irreversibly to central neurotransmitter receptors [34, 35]. Likewise, the chloramine presently studied, 8-methoxy-2′-chloro-PAT, should be useful for blocking irreversibly central 5-HT binding *in vivo*, and investigating both the associated functional consequences, and the turnover rates of the various classes of 5-HT sites already identified in brain (see [36] for review).

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