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"SPECIFIC BINDING" OF ^{3}H-PHENCYCLIDINE: ARTIFACTS OF THE RAPID FILTRATION METHOD

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Summary

Attempting to understand the nature of the specific binding of [^{3}H]-phencyclidine (^{3}H-PCP) to rat brain membranes, demonstrated recently with the rapid filtration method (I: Vincent et al., PNAS 76: 4678, 1979; II: Zukin and Zukin, ibid., 5372), we found that ^{3}H-PCP binds to GF/B glass fiber filters in a manner indistinguishable from "specific binding". The apparent Kd for this binding is 0.24 + 0.12 μM, not significantly different from the values in I and II. Binding of ^{3}H-PCP to the P_2 fraction of rat brain membranes is not affected by denaturing the tissues in boiling water. The apparent Kd and Bmax values are nearly the same for the native and denatured P_2 fractions. The definition of pharmacologically meaningful binding sites for ^{3}H-PCP by the rapid filtration method is further hampered by the fast dissociation rate of bound ^{3}H-PCP, as expected from the relatively large Kd. The amount bound is sensitive to the separation (washing) procedure: two volumes of 5ml reduce specific binding to native tissue by 60%, four volumes reduce it by 80%. We show that these characteristics of ^{3}H-PCP binding to both GF/B filters and denatured tissue are not shared by other radioligands, such as ^{3}H-D-lysergic acid diethylamide (^{3}H-D-LSD) and ^{3}H-(±)3-quinuclidinyl benzylate (^{3}H-QNB). The very large filter binding of ^{3}H-PCP is shown to be the reason for spurious differences in the density of PCP binding sites in various brain regions: we find that the amount of ^{3}H-PCP bound per assay is equal in each of the ten brain regions studied but that, due to differing amounts of protein in assays from different re-regions, an apparent regional distribution of binding sites is obtained when the results are normalized to binding per mg protein. The competition of other ligands for ^{3}H-PCP binding to all three materials - glass fiber filters, native and denatured P_2 fraction - yields nearly identical IC50 values and potency rankings. These values are in good agreement with I and II indicating that in this case the pharmacological identity criterion does not discriminate between receptor and nonreceptor interactions. We conclude that the artifacts created in this case by the rapid filtration method render it unsuitable for labeling the pharmacologically relevant binding sites of ^{3}H-PCP. If specific PCP receptors exist, as claimed in I and II, methods other than rapid filtration must be used to reveal them.

Many different mechanisms have been invoked to explain the unusual pattern of pharmacological activities elicited by Phencyclidine [N-(1-phenylcyclohexyl) piperidine, PCP] and its analogs (1-3). In binding experiments with tritiated 4-(N-methyl) piperidyl benzilate as the specific muscarinic

radioligand, PCP and some of its analogs were shown to compete for muscarinic binding sites (4). These studies demonstrated a good correlation between the antimuscarinic potencies measured for PCP and its derivatives in isolated organ preparations and the affinity of these drugs for the muscarinic binding sites in membranes from mouse brain (4). In related studies, quantum chemical calculations had identified an acetylcholine-like interaction pharmacophore in PCP (5,6) and this made possible the prediction of antimuscarinic potencies for novel PCP derivatives (7) which were subsequently synthesized (8). The antimuscarinic properties observed experimentally for these compounds (8) confirmed the ranking predicted theoretically from the ACh-like interaction pharmacophore (7). The binding of PCP and a large number of analogs to sites labeled by muscarinic antagonists was confirmed in binding studies with ^{3}H-QNB using membranes from rat brain (9,10) and from guinea pig ileum (10). It seems clear, however, that the muscarinic actions of PCP cannot explain the entire spectrum of pharmacological and behavioral responses elicited by PCP and its analogs (2). Recently, two different laboratories reported results on PCP binding to a class of distinct, specific PCP receptors in membranes from rat brain homogenates (11,12). These conclusions rest on direct binding experiments with ^{3}H-PCP using the rapid filtration method. From these studies PCP appeared to bind specifically with an affinity of 0.15 to 0.25 μM to sites that did not seem to bind putative neurotransmitters and neuromodulators.

These recent attempts (11,12) to characterize PCP binding by direct labeling of the sites with ^{3}H-PCP using a rapid filtration method are intriguing because it has long been assumed that for ligands with relatively low affinity (Kd > 0.1 μM) the rapid filtration method may not work at all (13). Moreover, in the two recently published reports (11,12), the competing ligand for the characterization of "nonspecific" binding was unlabeled PCP although it is usually accepted that in order to avoid artifacts it is preferable to use a masking ligand that is chemically different from the radiolabeled ligand (13).

In an attempt to understand the nature of the specific binding of PCP as reported (11,12) we performed a series of controls that constitute necessary and well established probes of the binding characteristics [e.g., see (14,15)]. We report here experiments that lead to the conclusion that the rapid filtration method as used recently (11,12) can produce artifacts in the characterization of pharmacologically relevant binding sites of ^{3}H-PCP.

Materials and Methods

Male rats (200-250g) were killed by decapitation, their brains removed, weighed and homogenized in 40 vol. ice cold 0.32M sucrose (10 strokes in Teflon pestle glass homogenizer and twice for 15 seconds in Polytron, setting #7). The homogenate was centrifuged for 10 min at 1000xg and the pellet discarded. The supernatant, S1, was centrifuged for 12 min at 40,000xg. The pellet (P_2) was washed by suspension in 40 volumes of 50mM Tris HCl (pH=7.4 at 0°C) followed by centrifugation at 40,000xg for 12 min. Two such washes were performed. The washed P_2 was resuspended in 10 volumes of the same buffer and was kept on ice until used.

Denaturation of tissue from this fraction was carried out by placing it in a boiling water bath for 5 min. Protein was determined according to Lowry et al. (16) with bovine serum albumin as standard. Protein contents stated for denatured tissue are the amounts before denaturation.

In a typical binding experiment, 1.0ml of 50mM Tris HCl (pH= 7.4, 0°C) containing the drugs was incubated in test tubes at 0°C for 30 min with and

without the P_2 fraction (0.3-0.5 mg protein/assay). The contents were poured onto GF/B filters (Whatman) mounted over vacuum. Usually the test tubes were washed once and the filters were washed twice with 3.0ml of 50mM Tris HCl (pH= 7.4, 0°C). When conditions in (12) were duplicated, washing was as described there. The radioactivity trapped on the filters was measured with 10ml of scintillation cocktail (formula 963 of New England Nuclear Co., Boston) using a Beckman LS900 with 43% efficiency.

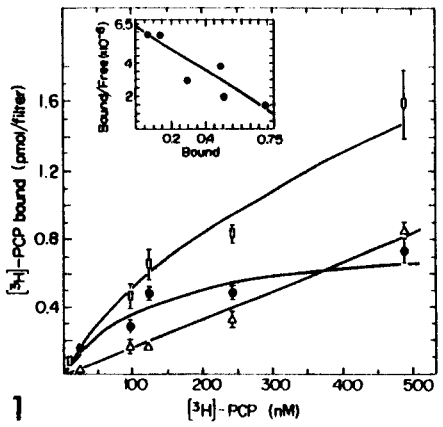
^{3}H-PCP was obtained from two different sources: New England Nuclear Co. (60 Ci/mmol, Lot #1031-202) which contained about 7% impurities, and from Dr. O. Buchman, Nuclear Research Center, Negev, Israel (19.0 Ci/mmol) with >99.5% chemical purity. The purity of the labeled compounds was analyzed on three TLC silicagel systems (250 μm): i) n-butanol/glacial acetic acid/water (150/24/60); ii) methanol/benzene (120/80); iii) methanol. The tritiated PCP from New England Nuclear Co. was purified with Sephadex ion exchanger (SP C-25, Pharmacia). Loading of the PCP and elution of the impurity were done with 5mM sodium phosphate pH= 7.4; the ^{3}H-PCP, identified by TLC, was eluted with 50mM of the same buffer. Most experiments were done with the 19 Ci/mmol ^{3}H-PCP diluted to 2 Ci/mmol.

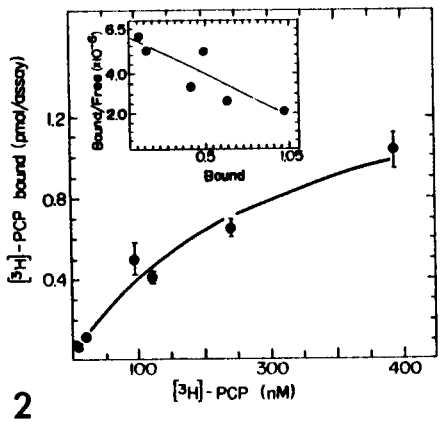
PCP, N-[1-(2-thienyl) cyclohexyl] piperidine (TCP), N-ethyl-1-phenyl cyclohexylamine (PCE), (1-[1-phenylcyclohexyl] morpholine (PCM) were obtained from NIDA. Atropine (sulphate hydrate) was bought from Calbiochem and (+) Ketamine from Brystol Comp., Syracuse. Triatiated (±) QNB (20 Ci/mmol) and D-LSD (13.3 Ci/mmol were bought from New England Nuclear Co. 5-Hydroxytryptamine (oxalate) and Tris buffer from Sigma Co.

Results and Discussion

Specific Binding of ^{3}H-PCP to Filters and to Native and Denatured Rat Brain Homogenate. In preliminary controls we found that ^{3}H-PCP binds to the GF/B glass fiber filters used in the rapid filtration method. In order to compare this finding with the reported results of saturation experiments (11, 12) we examined the binding to filters as a function of the concentration of ^{3}H-PCP using the experimental protocol as published (12). For the concentration range of ^{3}H-PCP used (12), the difference between the amount of labeled ligand bound to filters in the presence and absence of 100 μM unlabeled PCP is saturable and appears to have all the characteristics of "specific binding" (Figure 1): The analysis (performed on the PROPHET computer system) of the net binding curves (total binding minus binding in the presence of 100 μM PCP) yields an excellent hyperbolic fit to the Michaelis-Menten equation, a linear Scatchard plot (Fig. 1), and a Hill plot with slope not significantly different from unity. The binding parameters, Kd and Bmax, obtained from all three methods of analysis are in good agreement (Table 1). As expected from the relationship between the relatively low affinity of the radioligand (Kd >0.1 μM) and the allowable separation i.e., washing time (<0.1 sec. according to Table 1 in (13)), we observed unusually high scatter of the experimental results. All values reported are therefore averages from sextuplicate determinations. Because ^{3}H-PCP showed high adsorbance to almost any surface, each supporting grid (crucible) of the GF/B filter was used only once in an experiment.

The specific binding of tritiated PCP to the P_2 fraction (Figs. 2 and 3) was found to be very similar to the binding of ^{3}H-PCP by glass fiber filters alone (Fig. 1). The Kd values obtained from the hyperbolic fit and from the Scatchard plot are very nearly the same for ^{3}H-PCP binding to filters alone or to filters and native or denatured tissue (Table 1). Following the published experimental protocol (12) we found Bmax values (expressed as picomoles per assay) that are very similar for all three types of binding materi-





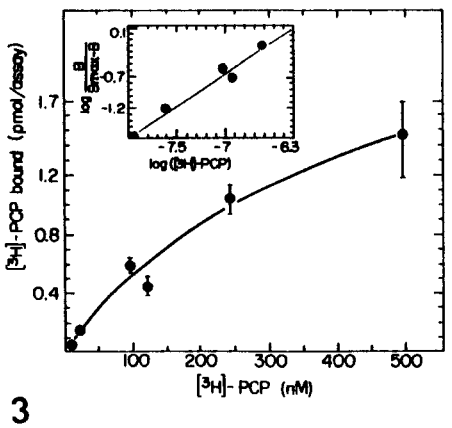


Fig. 1-3 Binding of [^{3}H]-PCP to GF/B filters (1), to native (2) and to denatured (3) P_2 fraction from rat brain, in the absence (□) and the presence (△) of 100 µM unlabeled PCP. (See Method and Materials Section for details). Insets are Scatchard (Figs.1 and 2) or Hill plots (Fig. 3) of specific binding (●). Points are the averages of sextuplicate determinations. Bars denote standard deviations. See Table 1 for binding parameters.

Table 1. Binding parameters of [^{3}H]-PCP to GF/B filters and native and denatured P_2 fraction from rat brain membranes.*

	Kd (µM)		Bmax (pmol/assay)		
Binding material	Hyperbolic fit	Scatchard plot	Hyperbolic fit	Scatchard plot	Hill Coefficient
GF/B filters	0.28±0.21	0.24±0.12	1.21±0.50	1.14±0.30	1.01±0.1
Native P_2	0.34±0.08	0.30±0.08	1.11±0.61	1.06±0.61	0.96±0.01
Denatured P_2	0.34±0.14	0.36±0.30	2.0 ±1.0	2.4 ±1.3	0.95±0.09

*Data are averages from two saturation experiments. In each saturation experiment binding to filters and to native and denatured tissue is determined in parallel with the same solutions and tissue preparations in sextuplicate determinations (0.4mg protein/assay).

als (Table 1). Results obtained with purified and nonpurified ^{3}H-PCP (60 Ci/mmol) were identical.

Specific Binding of [^{3}H]-PCP as a Function of Protein Content. To elucidate the relation between the apparently specific binding of [^{3}H]-PCP to filters and to material from rat brain homogenates (native as well as denatured) we analyzed the binding as a function of the protein content per assay. The protein content per assay used in the recent reports on ^{3}H-PCP binding differed greatly: 0.5mg (12), and 1.8 mg (11). Also different were the incubation temperatures: 4°C, and 25°C, respectively. In order to reproduce these conditions we studied the binding od ^{3}H-PCP in two ranges of protein concentration and at two different incubation temperatures, corresponding to the published work: i) 85nM ^{3}H-PCP with 0.03 to 0.30 mg protein per assay at 0°C; and ii) 100nM ^{3}H-PCP with 0.2mg to 1.45 mg protine per assay at 25°C.

The dependence of the amount of specifically bound ^{3}H-PCP on the amount of protein per assay is shown in Figure 4 for the first set of conditions described above. In the region between 0.03 and 0.2 mg protein the amount bound appears to be practically constant and nearly identical to the amount bound to filters alone. Even at higher concentrations the "specific binding" appears to be composed of at least 65 percent filter binding. Statistically, the experimental points fit a line ($r= 0.89$) with a slope of 0.87 and an intercept of 0.21. Thus ^{3}H-PCP binding does not extrapolate to zero at zero protein per assay: the intercept is significantly different from zero, $p= 0.007$. The extrapolated value of ^{3}H-PCP binding at zero protein is close to the amount bound to the GF/B filters when the same concentration of ^{3}H-PCP is used in the filter binding experiments (0.29±0.05 pmol, n=2). The protein dependence of ^{3}H-PCP binding to denatured tissue under the same experimental conditions yields results that are not different from those obtained with native material, according to the Friedman rank sums method test (17) in which binding values for each of the six replicates of each protein concentration were compared.

With the second set of conditions described above, the dependence of specific binding ^{3}H-PCP on protein amounts was also found to fit ($r= 0.88$) a line (Fig. 5) that does not extrapolate to zero (slope= 0.34; intercept= 0.3, the intercept is significantly different from zero $p= 0.06$). Here again the native and denatured materials exhibit the same behavior, and the intercept is not significantly different from the amount of specific binding to GF/B filters measured in separate experiments under the same conditions.

Comparison of ^{3}H-PCP Binding Characteristics to those of Other Radioligands. The characteristics of ^{3}H-PCP binding to both GF/B filters and denatured tissue are not shared by other radioligands used in binding studies with the rapid filtration method, such as ^{3}H-D-LSD or ^{3}H-QNB. We compared the characteristics of "specific binding" of radiolabeled PCP, LSD and QNB to the same tissue preparations, both native and denatured. Equal amounts of tissue were used in the assays for all ligands (0.29 mg protein per assay) but different incubation protocols were followed according to the conditions reported in the literature; i) 100 µM unlabeled PCP were used to define nonspecific binding of 80nM [^{3}H]-PCP incubated for 30 minutes at 0°C, according to (12); ii) 10 µM unlabeled 5-HT were used to define nonspecific binding of 0.6nM [^{3}H]-D-LSD incubated for 90 minutes at 25°C, according to (18); iii) 1 µM unlabeled atropine was used to define nonspecific binding of 0.6nM [^{3}H]-QNB incubated for 90 minutes at 25°C, according to (19). Results in Table 2 show that of the three ligands compared only [^{3}H]-PCP exhibits specific binding to both GF/B filters and denatured tissue. Earlier work has shown that the linear dependence of [^{3}H]-QNB (19) and [^{3}H]-D-LSD (20) binding on protein concentration extrapolates to zero specific binding at

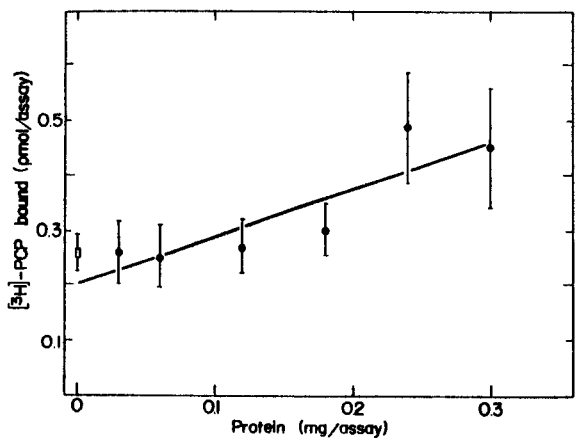


Fig. 4 Specific binding of [^{3}H]-PCP (85nM) to native P_2 fraction from rat brain homogenate under conditions described in (12). Points are the averages of sextuplicate determinations. See text for further details. Amount bound to filters in the absence of tissue (□) was calculated from a saturation experiment such as that shown in Fig. 1.

Table 2. Total and specific binding (fmols/assay) of different radioligands to the same binding materials.

Radiolabeled ligand	Type of binding	GF/B filters	Native tissue	Denatured tissue
PCP*	Total	290 ± 30	370 ± 30	550 ± 30
	Specific	240 ± 40	300 ± 30	400 ± 70
D-LSD†	Total	2 ± 0	17 ± 0	8 ± 0
	Specific	0	11 ± 1	0
QNB#	Total	1 ± 0	170 ± 6	3 ± 0
	Specific	0	168 ± 6	0

* 80 nM [^{3}H]-PCP/100 µM PCP, 0°C, 30 min.
† 0.6 nM [^{3}H]-LSD/10 µM 5-HT, 25°C, 90 min - Ref. 20.
# 0.6 nM [^{3}H]-QNB/1 µM atropine, 25°C, 90 min - Ref. 19.

zero protein content, but this is not so for ^{3}H-PCP (Figs. 4 and 5).

Artifacts of the Rapid Dissociation of Bound ^{3}H-PCP. The dissociation rate of bound [^{3}H]-PCP was estimated (11,12) from the rapid filtration experiments: full dissociation was observed (11) in less than 1 minute at 25°C, while at 0°C a dissociation constant of 0.48 min^{-1} was calculated by Zukin and Zukin (12). These kinetics are much faster than the observed binding rates of other radiolabeled ligands, including ^{3}H-QNB and ^{3}H-D-LSD, and should cause great difficulties in the use of the rapid filtration method: When tissue - containing filters are washed to remove the unbound [^{3}H]-PCP, a significant amount of "specifically bound" radioligand should also be displaced due to the fast dissociation rate. Figure 6 shows the effect of washing on the specific binding of three radiolabeled ligands: PCP, D-LSD and QNB. Only the "specific binding" of ^{3}H-PCP is affected by the washing procedure, and in the same manner for native and denatured tissue. The degree of uncertainty in the amount bound resulting from this spurious dissociation negates the validity of any determination of binding constants for ^{3}H-PCP with the rapid filtration method, for the values will be dependent on the washing procedure.

Regional Distribution of Specific ^{3}H-PCP Binding. We determined the amount of specific binding of 7nM ^{3}H-PCP in the P_2 fraction obtained from 10 different regions of rat brain (Table 3). Even though tissue was pooled from three rat brains the amount of protein obtainable from different regions was not equal: only 0.08mg protein were available per assay from the amygdala and the cervical spinal cord, and only cerebellum, medulla-pons, frontal cortex and hippocampus yielded P_2 fractions from which 0.34 mg protein could be used per assay. The experiments were carried out in triplicate rather than sextuplicate due to the small amount of tissue in some regions. We nevertheless considered it necessary to measure the regional distribution in the P_2 fraction rather than in whole homogenate [as in (12)] or in the S_1 fraction [as in (11)] because the characterization of ^{3}H-PCP binding was carried out in this fraction. It is well known that great differences can occur in radioligand binding characteristics of various fractions so that the choice of ^{3}H-PCP concentration used to assay regional distributions of binding is meaningful, with respect to the degree of receptor occupancy, only in the fraction that has been characterized (also see below).

Results in Table 3 show that the amount of ^{3}H-PCP bound per assay is the same in all regions and is practically identical in native and in denatured tissue. In agreement with results described above (Fig. 4) the average amount bound per assay is indistinguishable from the filter binding under the same conditions. A spurious regional distributions of binding appears (Table 3) if the results are normalized to the amount of protein and expressed as amount bound per mg protein. For example, the binding per assay is the same in the preparations from medulla-pons (105 $\pm$ 10) and from the amygdala (109 $\pm$ 14), but because the protein amount in the preparation from medulla-pons is much larger (0.34 mg) than in the amygdala preparation (0.08 mg), the normalization factor to binding per mg protein is 2.94 for medulla-pons and 12.5 for amygdala. This generates an artifactual difference in the "specific binding" by yeilding 309 $\pm$ 29 fmol/mg protein in the medulla-pons and 1362 $\pm$ 175 fmol/mg protein in the amygdala (Table 3). This spurious regional distribution is similar for native and denatured tissue. The regional distribution of ^{3}H-PCP binding reported recently (11,12) could well be a result of the normalization artifact described here. This might also explain the totally different amounts of binding in regions and totally different regional distribution found in comparing these two reports (11,12).

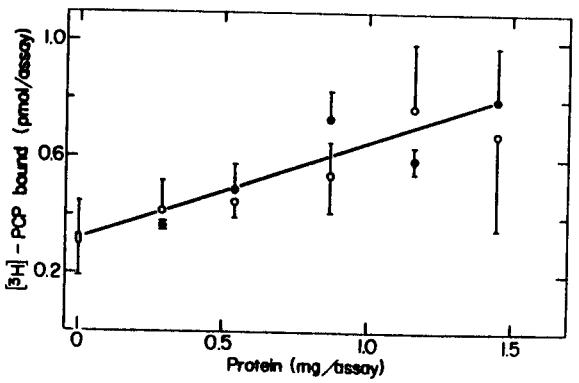


Fig. 5 Specific binding of 87nM [^{3}H]-PCP to native (○) and denatured (●) P_2 fraction from rat brain homogenate, under conditions described in (11). See text for further details. In a control experiment, run in parallel, no tissue was included (□). Values shown are the average of sextuplicate determinations.

Table 3

Regional Distribution of ^{3}H-PCP Binding

Region[1]	mg prot assay	Native		Denatured	
		fmol/assay	fmol/mg	fmol/assay	fmol/mg
Cerebellum	0.34	61 ± 9	179 ± 26	82 ± 7	241 ± 20
Medulla pons	0.34	105 ± 10	309 ± 29	56 ± 15	165 ± 43
Frontal Cortex	0.34	76 ± 6	223 ± 18	72 ± 8	211 ± 23
Cingulate Cortex	0.23	80 ± 12	347 ± 52	101 ± 19	439 ± 83
Caudate Nucleus	0.21	76 ± 11	361 ± 52	59 ± 8	281 ± 38
Hippocampus	0.34	110 ± 31	323 ± 91	77 ± 9	226 ± 25
Amygdala	0.08	109 ± 14	1362 ± 175	99 ± 11	1237 ± 137
Subiculum	0.22	83 ± 3	377 ± 14	64 ± 20	290 ± 91
Hypothalamus	0.17	60 ± 24	354 ± 141	62 ± 22	369 ± 129
Cervical spinal cord	0.08	49 ± 9	612 ± 112	95 ± 23	1187 ± 287
Mean	-	81 ± 21	-	77 ± 17	-
Filters	91±5				

1) Pooled from 3 rat brains. See text and Materials and Methods section for details.

Effects of other Drugs on the Specific Binding of ^{3}H-PCP. One criterion used to determine the pharmacological relevance of the specific binding of radiolabeled drugs rests on the identity of the drugs that compete with the binding of the labeled ligand. It is expected (14) that the pharmacology of binding (i.e., the concentrations at which the competing ligands are effective, and the rank order of their potencies as competitors) will correlate with the pharmacology of receptor mediated effects. In the recent publications (11,12), the competition of PCP analogs with [^{3}H]-PCP binding was considered to reflect such a receptor related pharmacological specificity. To test the validity of these conclusions we carried out competition experiments, using the same conditions as described in (12), on the three types of binding materials: GF/B filters, native tissue from the P_2 fraction, and denatured tissue from the same homogenate preparation. Table 4 shows that the concentrations needed to inhibit 50 percent of ^{3}H-PCP binding, as well as the rank order of potencies of the competing ligands are in good agreement for all binding materials, including filters and denatured tissue. Again, a good agreement is obtained with the published data (11,12) suggesting that the pharmacological identity criterion is not operative in this case, probably due to the artifacts inherent in the application of the rapid filtration method to ^{3}H-PCP binding. It is important to note that the competition experiments (and the regional distribution assay described above) were carried out with exceedingly low concentrations of ^{3}H-PCP, as used in the recent reports (11,12). This was done for the sake of comparison but does not represent a generally recommendable procedure: from the Kd values published (11,12) it follows that the 7nM ^{3}H-PCP used in (12) would occupy only 4.5 percent of the receptors, whereas the 1nM ^{3}H-PCP used in (11) would occupy only 0.4 percent of the receptors. These occupancies may be much too small to establish reliable ligand competition relationships or to probe the nature and distribution of binding sites. In fact, 1nM ^{3}H-PCP is much lower than the lowest concentration used in determining the saturation curve in (11), and 7nM ^{3}H-PCP is the lowest point on the saturation curve in (12).

Concluding Remarks

The popularity of the rapid filtration method for binding studies with radiolabeled ligands is reflected not only in its documented successes in the characterization of pharmacological receptors but also in the increasing number of descriptions of pitfalls and possible artifacts encountered in its use (21). The controls and criteria used in the distinction of receptor from non-receptor interactions were recently summarized and reviewed (15,21). It is shown, for example, that spurious "specific binding" to glass fiber filters such as demonstrated here for ^{3}H-PCP also occured with other labeled ligands (e.g., ^{125}I-glucagon, ^{125}I-insulin, ^{3}H-naloxone) to a variety of inert materials including glass tubes, talcum and millipore filters. It is also shown in these reviews (15,21) that labeled drugs have been found to interact with natural substances that are not likely to be active pharmacological receptors (e.g., serum proteins) with relative affinities reflecting potencies in vivo. This is similar to the binding of [^{3}H]-PCP to denatured tissue, and to the ability of drugs to compete for the "specific ^{3}H-PCP binding" sites on filters and denatured P_2 fraction.

The controls performed here to characterize ^{3}H-PCP binding may be helpful, as suggested earlier (15,21), in identifying some of the possible artifacts of the rapid filtration methods related to the use of a charged, lipophilic radioligand with a low affinity for biological receptors. It is quite clear that the extent of net binding to filters must be assessed and that the linear dependence of specific binding on the amount of protein per assay has to be tested for its extrapolation to zero binding at zero tissue. The sensitivity of specific binding to the experimental protocol (e.g., washing) and to protein denaturating conditions (e.g., boiling) have to be determined

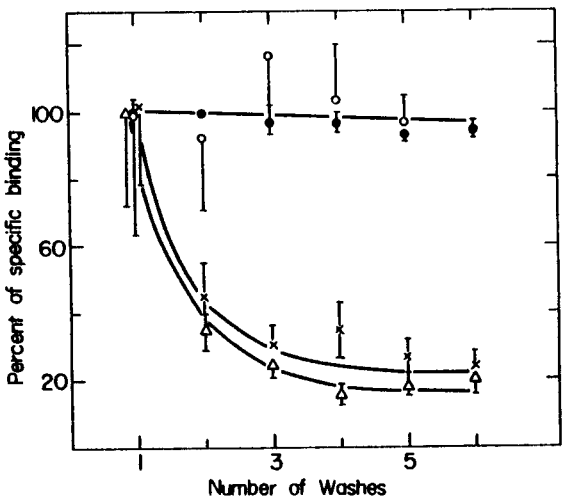


Fig. 6 Effect of the separation procedure (number of washes with 5ml buffer) on the amount of specific binding of 100nM [^{3}H]-PCP (△), 4.5nM [^{3}H]-D-LSD (○), 1nM [^{3}H]-QNB (●) to native P_2 fraction from rat brain (0.3mg protein/assay), and 100nM [^{3}H]-PCP to denatured P_2 fraction (X). See text and legend to Table 2 for incubation conditions. The specific binding for each ligand after one washing with 5ml buffer (1.2, 0.04, 0.2, and 1.2 pmol/assay, respectively) was taken as 100 percent. Vertical bars indicate standard deviations from sextuplicate (PCP) or triplicate (LSD, QNB) determinations.

Table 4 Relative potencies of drugs in reducing [^{3}H]-PCP binding to various materials.

Drug	IC_{50}*, μM				
	GF/B filters	Native tissue	Denatured tissue	Native¶ tissue	Native§ tissue
PCP†	0.1	0.1	0.2	0.23	0.25
TCP†	0.15	0.06	0.08	0.16	0.026
PCE†	2	1	1	0.14	ND
PCM#	1	5	6	2.4	ND
Ketamine#	100	100	100	1	7
Atropine#	90	150	150	1000	> 10

* Concentrations reducing the specific binding of 10 nM [^{3}H]-PCP to 50 percent of control.
† Results are interpolated from a nine point competition curve with unlabeled ligand concentrations ranging from 10 nM to 100 μM.
# Results are interpolated from a nine point competition curve with unlabeled ligand concentrations ranging from 100 nM to 1mM.
¶ Ref. 12
§ Ref. 11 (ND- not determined)

(21). The importance of performing such controls at concentrations of the labeled ligand that are relevant to its saturation curve (i.e., yielding close to 50 percent occupancy), but mindful of the necessary large ratio between ligand concentration and receptor concentration has also been established (15). The results of such controls performed here for ^{3}H-PCP binding to the P_2 fraction from rat brain homogenates indicate that to the extent that binding sites were identified recently using the rapid filtration method (11,12), they do not fulfill the criteria for discrimination between receptor and nonreceptor interactions (15). It is interesting to note that the physicochemical parameters that determine the equal rank order of binding of PCP derivatives to GF/B filters and to native and denatured tissue are also related to the activity of these compound in the rotarod test. The correlation observed recently (11,12) between ^{3}H-PCP binding and rotarod activity may be a reflection of this relation for it is well known that pharmacological activity often correlates well with the physicochemical properties of drugs such as their pKa values or their oil/water partition coefficients.

Our conclusions regarding the artifacts of the rapid filtration method that prevent it from yielding meaningful results for ligands such as ^{3}H-PCP do not preclude using other methods (e.g., dialysis, centrifugation) to separate tissue bound ^{3}H-PCP from the free radiolabeled ligand in experiments designed to characterize of high affinity binding sites of PCP.

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