Biochemical Pharmacology, Vol. 33, No. 24, pp. 4039–4044, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

CHARACTERIZATION OF CHOLATE-SOLUBILIZED DOPAMINE RECEPTORS FROM HUMAN, DOG AND RAT BRAIN

WALTER WOUTERS, JACKY VAN DUN and PIERRE M. LADURON
Department of Biochemical Pharmacology, Janssen Pharmaceutica, B-2340 Beerse, Belgium

(*Received* 18 *April* 1984; *accepted* 11 *July* 1984)

Abstract—[^{3}H]Spiperone binding sites were solubilized in high yield from human, dog and rat brain with a mixture of sodium cholate (0.3% w/v) and sodium chloride (1.4 M). The binding sites were not sedimented after one hour at 100,000 *g*, they passed freely through 0.20 μm filters, migrated as a single peak in gradient sedimentation and were retarded upon gel filtration, proving that they were truly solubilized. The solubilized binding sites were definitely of dopaminergic nature. They showed saturable, reversible, high affinity binding of [^{3}H]spiperone; displacement of [^{3}H]spiperone binding by nanomolar concentrations of dopamine antagonists and micromolar concentrations of serotonin antagonists; stereospecificity and a good correlation with drug affinities for membrane preparations. The non-displaceable, non-specific [^{3}H]spiperone binding was very low.

Gradient sedimentation analysis revealed a sedimentation coefficient of 12 S for dog solubilized preparations, 9 S for rat solubilized preparations and only 2.5 S for human solubilized preparations (values, uncorrected for detergent binding). Gel filtration experiments seem to confirm these molecular characteristics.

Therefore the present results show that the dopamine receptor reveals the same pharmacological properties when solubilized with cholate-salt from rat, dog or human brain, while physico-chemical properties seem to indicate some differences.

For many years, neuroleptic drugs are known to interact with dopamine receptors in the brain; they have been successfully used as ligands to identify and characterize the dopamine receptor (D_2-subtype) through *in vitro* binding assays. Solubilization and purification of the receptor site are both prerequisite before determining its chemical structure and further elucidating the functional aspects of agonist and antagonist binding.

A first attempt to solubilize dopamine receptors from rat brain using digitonin was reported by Gorissen and Laduron [1]; however, a very high non-specific and displaceable binding on sites which were identified as spirodecanone sites [2] masked the binding on dopamine receptors [2, 3]. The first solubilization of high affinity dopamine receptors was obtained from dog brain using 1% digitonin [3, 4]; later on this was confirmed in other animal species: rat, calf and human brain, using digitonin or other detergents such as lysophosphatidylcholine and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate) [5–9]. However, most of these procedures gave either a low solubilization yield or a too high non-specific binding.

More recently, Hall *et al.* [10] described a method based on the use of sodium cholate (0.2%) and sodium chloride (1 M); under these conditions a higher yield of solubilized dopamine receptors was obtained from calf striatal membranes and the non-specific binding was relatively low.

We now report on the use of a cholate-salt mixture to solubilize dopamine receptors from human, dog and rat brain and on the molecular characteristics of these sites.

MATERIALS AND METHODS

Brain membrane preparation

Mongrel dogs were anaesthetized with pentobarbital and Wistar rats were decapitated; their brains were removed and striata rapidly dissected out. They were homogenized in 0.25 M sucrose and a microsomal (P) fraction was prepared as described by Laduron [11]. The microsomal fraction was suspended in 2 vol. ice-cold water and kept at −16°.

Post-mortem human brains, without any sign of neurological disease were obtained 3 to 10 hr after death (Cliniques Universitaires Saint-Luc, U.C.L., Brussels). Putamen and caudate nucleus were removed and immediately put on ice. The microsomal fraction was prepared as above, suspended in 1 vol. ice-cold water and kept at −16°.

Solubilization procedure

One millilitre of microsomal fraction (corresponding to 0.5 g of fresh tissue for dog and rat brain, or 1 g of tissue for human brain) was mixed with 1 ml of solubilization buffer, containing 20 mM sodium phosphate pH 7.2, 0.5 M sucrose, 2.8 M sodium chloride and 0.6% (w/v) sodium cholate (Sigma C1254). After incubation at 4° for 12 min the mixture was diluted 1:2 with distilled water (the sodium chloride concentration becoming equal to 0.7 M) and immediately centrifuged at 120,000 *g* (r_{av}) for 60 min in a Beckman SW65Ti rotor. The supernatant was removed without disturbing the pellet and diluted 1:2 with distilled water. This final dilution was used as the soluble preparation.

Binding assays

Human membrane preparation. One millilitre of microsomal membrane preparation was washed with 25 vol. of 50 mM Tris–HCl, pH 7.6 containing 120 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride and 0.1% (w/v) ascorbic acid. Membranes were centrifuged at 23,000 *g* (r_{av}) for 20 min and the pellet was suspended in 100 vol. of the same buffer using a glass–Teflon homogenizer. Aliquots of this suspension were incubated for 15 min at 37° with 1 nM [^{3}H]spiperone (N.E.N. 23.8 Ci/mmole) and various concentrations of unlabelled drugs. They were then filtered under suction through Whatman GF/B glass fiber filters. Filters were washed twice with 5 ml of ice-cold buffer and transferred to counting vials. Insta-Gel II (Packard) was added and the vials were counted in an Intertechnique SL 4000 liquid scintillation counter.

Soluble preparations. Aliquots of the soluble preparation were incubated for 15 min at 25° with 1 nM [^{3}H]spiperone and various concentrations of unlabelled drugs. Bound and free ligand were separated using the charcoal method as described by Ilien *et al.* [5]. Aliquots of the supernatant were counted in Insta-Gel II. Specific binding was defined as that displaceable with 10^{-6} M (+)-butaclamol.

Sucrose gradient centrifugation

An aliquot (1 ml) of the soluble preparation was mixed with marker enzymes and layered on a 20–30% linear sucrose gradient containing 10 mM sodium phosphate, pH 7.2 and 0.4 M potassium chloride (11 ml total volume). The gradients were centrifuged at 4° in a Beckman SW40Ti rotor for 16 hr at 35,000 rev/min. After centrifugation, the gradient was collected in fractions of 0.75 ml and specific [^{3}H]spiperone binding, proteins and marker enzymes were determined in each fraction.

Gel filtration

Three millilitres of solubilized preparation were labelled with [^{3}H]spiperone as described above, mixed with marker enzymes and applied to a column of Sepharose CL-4B (360 × 26 mm) equilibrated with 10 mM sodium phosphate, pH 7.2, 50 mM sucrose, 0.4 M potassium chloride and 0.04% asolectin (American Lecithin Company, Atlanta) at 4°. Proteins were eluted with the same buffer at a constant flow rate of 20 ml/hr and 40-drop fractions (2 ml) were collected. [^{3}H]Spiperone, proteins and marker enzymes were determined in each fraction. The void volume (V_o) and total volume (V_t) were determined using dextran blue and potassium dichromate respectively.

Protein and enzymatic assays

Protein was estimated by the Bio-Rad method using bovine serum albumin as the standard. Lactate dehydrogenase from rabbit muscle and malate dehydrogenase from pig heart (Boehringer) were assayed by following the optical density at 340 nm after mixing with NADH in sodium phosphate buffer with sodium pyruvate or oxaloacetate as the substrate. Catalase from beef liver (Boehringer) was assayed by following the optical density at 240 nm after mixing with hydrogen peroxide in sodium phosphate buffer. Cytochrome C from horse heart (Sigma C2506) and ferritin from horse spleen (Boehringer) were assayed by their absorption at 530 and 350 nm, respectively.

Microfiltration assay

One millilitre of solubilized preparation was taken up in a plastic syringe with Luer tip. It was then pressed through a 0.20 μm Acrodisc disposable filter (Gelman). The filtrate was used for [^{3}H]spiperone binding and protein determination as described above.

RESULTS

Solubilization. Figure 1 shows the influence of the sodium cholate concentration on the amount of specific [^{3}H]spiperone binding sites and protein solubilized from rat and dog striata. A maximal specific [^{3}H]spiperone binding was obtained at a cholate concentration of 0.3% (w/v) for dog striata and between 0.3 and 0.4% (w/v) for rat striata. Protein reached a plateau value between 0.4 and 0.5% (w/v). For convenience a concentration of 0.3% (w/v) was chosen for further experiments with both species. The yield in solubilized [^{3}H]spiperone binding sites was also dependent on sodium chloride concentration (data not shown). The amount of solubilized binding sites increased rapidly up to a concentration of 1.4 M sodium chloride and then reached a plateau value. The concentration of 1.4 M sodium chloride was therefore adopted for further experiments. Solubilization was not possible using either sodium cholate or sodium chloride alone. In order to be sure that all membrane fragments would be spun down during centrifugation, the solubilization medium was further diluted 1:2 to reach a final concentration of 0.7 M sodium chloride for centrifuging. For human brain solubilization experiments, the same concentration of 0.3% (w/v) in

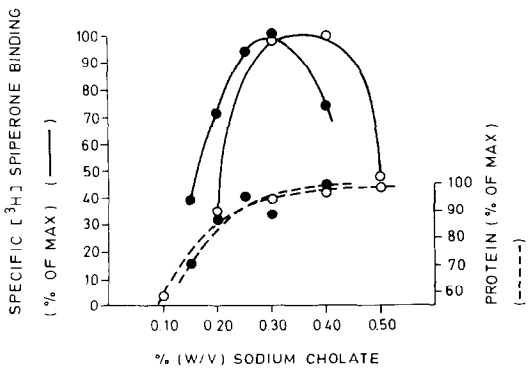

Fig. 1. Effect of increasing sodium cholate concentrations on the solubilization of specific [^{3}H]spiperone binding sites (——) and protein (---) from dog (●) and rat (○) brain. Membrane preparations were incubated with various concentrations of sodium cholate and 1.4 M sodium chloride at 4° during 12 min. Centrifugation and [^{3}H]spiperone binding were as described in the Methods section. Maximum solubilization of [^{3}H]spiperone binding sites occurred at 0.30% (w/v) sodium cholate for dog brain and 0.36% (w/v) for rat brain.

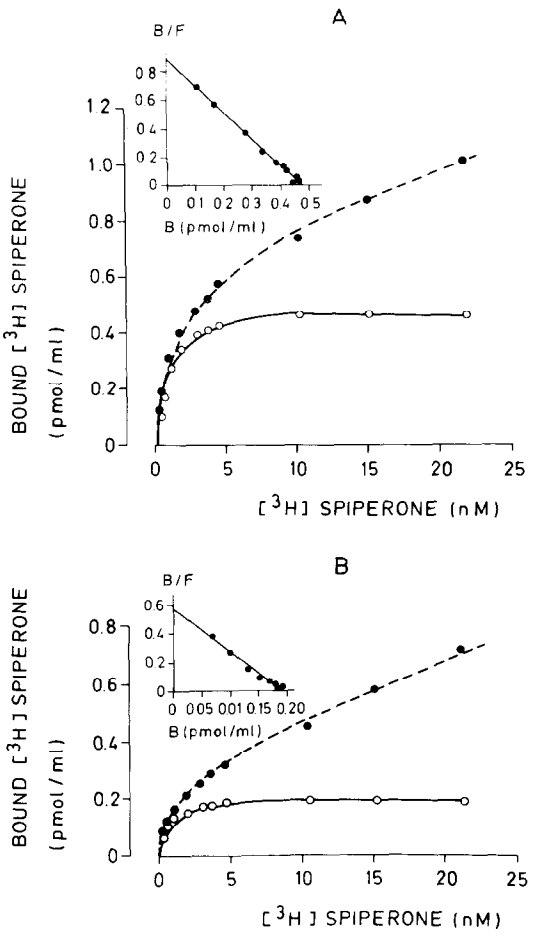

Fig. 2. Typical saturation curves for [^{3}H]spiperone binding to cholate solubilized preparations from dog (A) and human (B) brain. Solubilized preparations were incubated with increasing concentrations of [^{3}H]spiperone. Specific binding was determined using a 1000-fold excess of (+)-butaclamol. Inserts show Scatchard plots of the specific [^{3}H]spiperone binding. (●), total binding; (○); specific binding.

sodium cholate and 1.4 M in sodium chloride were also used. In order to obtain approximately the same protein/cholate ratio, the microsomal fraction was used at a concentration, twice that of rat and dog brain.

Soluble preparations of all three species were filtered through 0.20 μm filters. More than 90% of protein and specific [^{3}H]spiperone binding sites were recovered in the filtrate.

[^{3}H]*Spiperone binding*. Figure 2 shows saturation curves for [^{3}H]spiperine binding in soluble preparations from dog and human brain. Specific [^{3}H]spiperone binding was saturable in the nanomolar range. Scatchard analysis showed straight lines, indicating a single class of binding sites. Table 1 summarizes K_D and B_{max}-values for soluble preparations and corresponding membrane preparations from the three species. There was a good correlation of K_D-values between membrane and soluble preparations. B_{max}-values were used to calculate solubilization yields; they are also summarized in Table 1. The yields were 2 to 3 times higher than previously reported using other detergents. For human preparations the solubilization yield showed larger variations than for the other two species.

Specific [^{3}H]spiperone binding was reversible and could be dissociated at 25° by a 1000-fold excess of unlabelled dopamine antagonists as well as by a dopamine agonist, 2-(*N*,*N*-dipropyl)-amino-5,6-dihydroxytetralin at a concentration of 10^{-5} M.

Pharmacological characterization. Table 2 summarizes the IC_{50}-values for several compounds in competition for [^{3}H]spiperone binding to solubilized preparations and corresponding membrane preparations from human, dog and rat striata. Spiperone, (+)-butaclamol and benperidol competed in the nanomolar range. Stereospecificity was pronounced, the ratio for (+) and (−)-butaclamol being higher than 1000-fold. Pipamperone, a butyrophenone with antiserotonergic properties and R 5260, a compound possessing the spirodecanone moiety but completely devoid of dopaminergic activity [3] were only active in the micromolar range.

Table 1. Equilibrium parameters derived from the Scatchard analysis and solubilization yields for cholate solubilization of dopamine receptors from dog, rat and human brain

	K_D (nM)	B_{max} (pmole/mg protein)	B_{max} (pmole/g tissue)	Solubilization yield (%)
Dog membrane*	0.25	0.77	15.40	—
cholate solubilized preparation‡	0.55 ± 0.02	0.75 ± 0.13	8.14 ± 1.36	53 ± 9
Rat membrane†	0.42	1.70	23.76	—
cholate solubilized preparation‡	0.54 ± 0.01	1.14 ± 0.15	10.81 ± 1.42	46 ± 6
Human membrane‡	0.22 ± 0.07	1.67 ± 0.65	4.06 ± 0.78	—
cholate solubilized preparation‡	0.33 ± 0.02	0.52 ± 0.01	2.02 ± 0.14	52 ± 13

* Values taken from [2].
† Values taken from [12].
‡ Values are means ± S.E.M. of 2 experiments performed in triplicate.
Solubilization yield was calculated as [B_{max} soluble (pmole/g tissue) ± S.E.M.]/[B_{max} membrane (pmole/g tissue) ± S.E.M.].

Table 2. IC_{50}-values (nM) for competition of 12 compounds in [^{3}H]spiperone binding to dog, rat and human membrane preparations and cholate solubilized preparations (values are uncorrected for receptor occupancy by [^{3}H]spiperone)

Drug	Dog		Rat		Human	
	Membrane*	Soluble	Membrane†	Soluble	Membrane	Soluble
Spiperone	1.8	2.5	1.3	1.8	0.6	3.0
Benperidol	5.1	4.0	4.5	3.8	1.8	5.0
(+)-Butaclamol	15	4.0	20	7.6	1.6	5.1
Haloperidol	16	45	20	5.2	7.9	52
Domperidone	22	23	20	20	7.6	22
Flupenthixol	40	32	59	50	5.6	79
Chlorpromazine	42	112	40	141	22	—
2-(*N*,*N*-dipropyl)-amino-5,6-dihydroxytetralin	110	178	130	575	126	575
(±)-Sulpiride	562	2951	871	1620	224	3020
Pipamperone	1288	2818	1300	2510	794	3020
(−)-Butaclamol	12,882	6309	2100	17,800	11,220	7943
R 5260	100,000	3467	20,000	6310	3020	6025

* Values taken from [3].
† Values taken from [3, 12].

Figure 3 shows the competition curves for these compounds in human and dog brain. The curves are parallel to each other and show a monophasic shape. The level of the non-specific binding is very low in dog brain and only slightly higher in human brain; there is no displacement below the threshold of non-specific binding.

Correlation between solubilized and membrane preparations was very good and gave Spearman rank values of $r = 0.963$ ($P < 0.001$, $N = 12$) for dog brain, $r = 0.979$ ($P < 0.001$, $N = 12$) for rat brain and $r = 0.961$ ($P < 0.001$, $N = 11$) for human brain.

Figure 4 shows a good correlation between IC_{50}-values in solubilized preparations from the three different species. The Spearman rank correlations were $r = 0.986$ ($P < 0.001$, $N = 11$) for dog/human, $r = 0.970$ ($P < 0.001$, $N = 11$) for rat/human and $r = 0.946$ ($P < 0.001$, $N = 12$) for dog/rat (data not shown).

Gradient sedimentation. Figure 5 shows gradient sedimentation profiles for the three species. In rat and dog solubilized preparations, specific [^{3}H]spiperone binding was recovered around catalase, with a sedimentation coefficient estimated to be 12 S in dog and 9 S in rat. Specific [^{3}H]spiperone binding from human solubilized preparations, however, was recovered before malate dehydrogenase with a sedimentation coefficient of about 2.5 S (sedimentation coefficients are uncorrected for detergent binding).

Gel filtration. Figure 6 shows gel filtration of cholate solubilized preparations from dog and human brain on a calibrated column of Sepharose CL-4B. Inclusion of crude soybean phospholipids (asolectin) in the eluting buffer was necessary to avoid dissociation of [^{3}H]spiperone from the receptor during elution. For human preparations, however, dissociation could not be inhibited completely, resulting in a second peak of free [^{3}H]spiperone eluting at the total volume (V_t). The insert of Fig. 6 shows the calibration curve for the column. Stokes radii of 3.42 and 5.65 nm were found for human and dog preparations respectively.

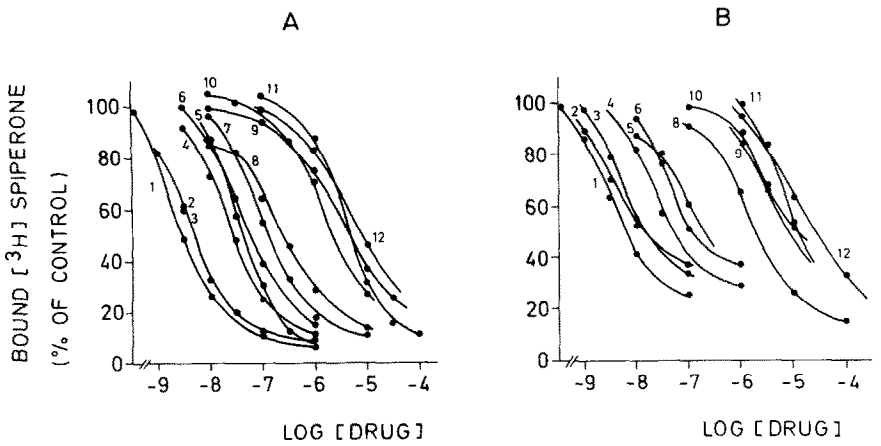

Fig. 3. Displacement of [^{3}H]spiperone binding to cholate solubilized preparations of dog (A) and human (B) brain by 12 drugs belonging to different classes. 1, spiperone; 2, benperidol; 3, (+)-butaclamol; 4, domperidone; 5, flupenthixol; 6, haloperidol; 7, chlorpromazine; 8, 2-(*N*,*N*-di-propyl)-amino-5,6-dihydroxytetralin; 9, (±)-sulpiride; 10, pipamperone; 11, R 5260; 12, (−)-butaclamol.

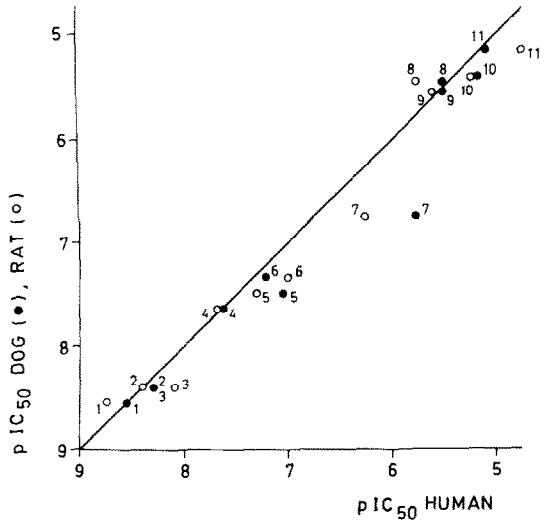

Fig. 4. Correlation between drug affinities for displacement of [^{3}H]spiperone binding in cholate solubilized preparations of dog/human (●) Spearman rank correlation $r = 0.986$ ($P < 0.001$, $N = 11$) and rat/human (○) Spearman rank correlation $r = 0.970$ ($P < 0.001$, $N = 11$). 1, spiperone; 2, benperidol; 3, (+)-butaclamol; 4, domperidone; 5, flupenthixol; 6, haloperidol; 7, 2-(*N*,*N*-dipropyl)-amino-5,6-dihydroxytetralin; 8, (±)-sulpiride; 9, pipamperone; 10, R 5260; 11, (−)-butaclamol.

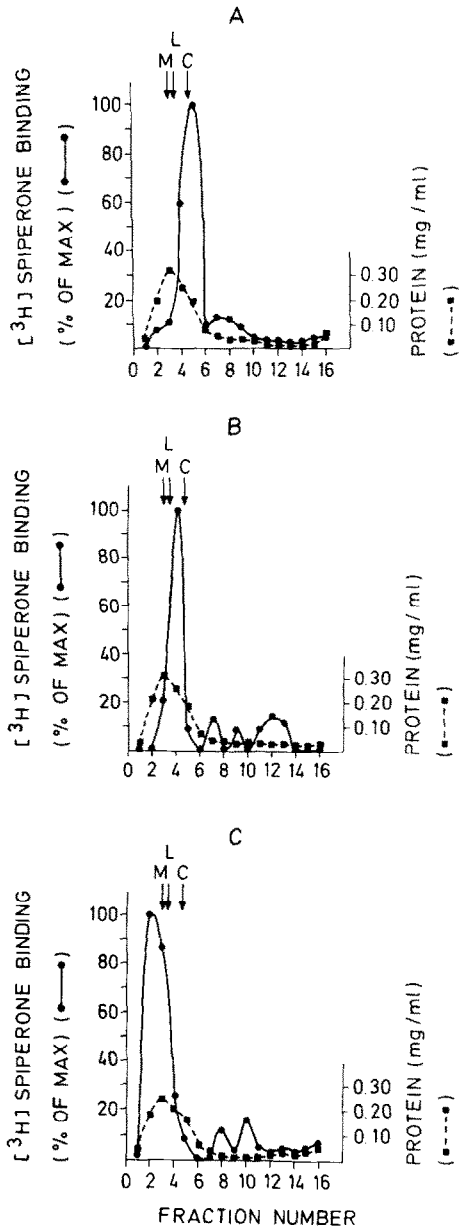

Fig. 5. Sedimentation profile for [^{3}H]spiperone binding sites (-●-●-) and protein (-■-■-) of cholate solubilized preparations from dog (A), rat (B) and human (C) brain on a 20–30% linear sucrose gradient. Marker enzymes are: M, malate dehydrogenase; L, lactate dehydrogenase and C, catalase.

DISCUSSION

D_2-Dopamine receptors were solubilized from dog, rat and human brain using a mixture of cholate and salt. Cholate is not only cheaper than most other detergents but also has the advantage of being easily dialysable.

Solubilization yields were two to three times higher than reported using other detergents. For human brain samples, this yield, however, showed rather large variations, depending on the way the fresh tissue was treated. It was necessary to use the human post-mortem samples as soon as possible after death. They could eventually be kept on ice overnight. Freezing of the tissue was absolutely to be avoided since this led to a decrease in solubilization yield with more than 50%.

The cholate solubilized preparations of the three species tested showed only one class of [^{3}H]spiperone binding sites. Displaceable non-specific [^{3}H]spiperone binding on spirodecanone sites [2] was totally absent; this is certainly one of the most interesting advantages of using cholate as the solubilizing agent.

Pharmacological analysis of the solubilized preparations indicates clearly that the [^{3}H]spiperone binding sites are of dopaminergic nature. Dopamine antagonists displaced [^{3}H]spiperone binding in the 10^{-8} M range and the tetralin derivative, a highly potent dopamine agonist, also showed high affinity towards the solubilized receptors. Pipamperone, an anti-serotoninergic compound, displaced only in the micromolar range. Moreover, the displacement of [^{3}H]spiperone by pipamperone showed a monophasic curve, indicating that no serotonin receptors were present. This is in contrast with the findings of Hall *et al.* [10] who found serotoninergic as well as dopaminergic sites in a cholate solubilized preparation from bovine brain. Therefore, the brains of the three species tested in the present work, should provide a better source of dopamine receptors for purification than does bovine brain.

In contrast to the similarities found upon pharmacological analysis of the three solubilized preparations, physico-chemical analysis seems to indicate some differences. Gradient sedimentation clearly

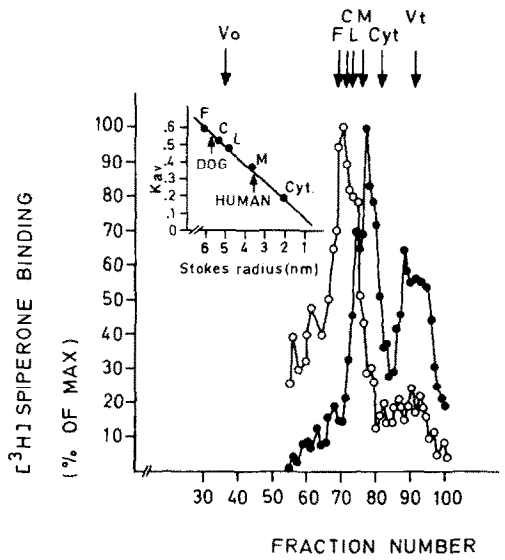

Fig. 6. Elution pattern for [^{3}H]spiperone binding sites of cholate solubilized preparations from dog (-○-○-) and human (-●-●-) brain, upon gel filtration on a calibrated column of Sepharose CL-4B. Insert shows the calibration curve of Stokes radii vs K_{av} [$K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)}$; V_e = elution volume, V_o = void volume and V_t = total volume]. Marker enzymes are: F, ferritin; C, catalase; L, lactate dehydrogenase; M, malate dehydrogenase; Cyt, cytochrome C.

showed differences in sedimentation coefficients, with values of 12 S, 9 S and 2.5 S for dog, rat and human solubilized preparations, respectively (values, uncorrected for detergent binding). Stokes radii, found upon gel filtration seem to confirm these differences. It is noteworthy that for gel filtration experiments, the inclusion of phospholipids in the eluting buffer was absolutely necessary, apparently to ensure stability of the receptor–ligand complex. This is in agreement with the observations of Hall *et al.* [10] who reported the use of phospholipids in gel filtration and density gradient sedimentation of cholate soluble preparations from bovine brain. Human soluble preparations could not be stabilized completely and gave rise to a peak of free [^{3}H] spiperone at the total volume.

Assuming a globular state for the solubilized receptor–detergent–phospholipid complex, molecular weights of ±200,000–300,000 D for dog and rat and of only ±60,000 D for human preparations can be derived. There are several possible ways to explain this apparent difference. Since the dopamine receptor is solubilized together with phospholipids and surrounding proteins, a difference in membranous environment could lead to molecular differences of the solubilized preparation. Secondly, the results obtained upon molecular characterization, could reflect a real difference between the size of human brain dopamine receptor and dopamine receptors from dog and rat brain. However, this would be in contrast with the results of Lilly *et al.* [13] who found the same molecular weight for human and dog brain D_2-receptors by radiation inactivation. Finally, receptor degradation due to the slow cooling of human brain tissue cannot be excluded. However, in experiments in which rat brain was treated in the same way as human brain, we found no differences with "normally" treated rat brain (unpublished results).

It will be interesting to find out what is the exact explanation for the molecular differences between solubilized dopamine receptors from dog, rat and human brain. In the meantime cholate solubilization offers a useful tool for further purification and characterization of these receptors.

Acknowledgements—We thank J. M. Maloteaux for providing us with the post-mortem human brain samples. Part of this work was supported by a grant from I.W.O.N.L.

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