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Physical separation and characterization of two types of benzodiazepine receptors

(diazepam/receptor heterogeneity/differential solubilization/β-carbolines/γ-aminobutyric acid)

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ABSTRACT **Two distinct benzodiazepine receptors are solubilized differentially by various detergents. The receptor sites that resist solubilization, designated type I, are most highly concentrated in the cerebellum and corpus striatum whereas the more readily solubilized receptors, type II, are most enriched in the hippocampus. The type I receptors display higher affinity for β-carboline esters and a triazolopyridazine whereas several benzodiazepines do not differentiate the two receptors. The type I receptors can be solubilized with 2% Triton X-100/1 M NaCl; they retain the same drug specificity as in the particulate state.**

The diverse effects of benzodiazepines may involve distinct subpopulations of benzodiazepine receptors (1, 2). For instance, CL-218872, a triazolopyridazine, potents at benzodiazepine receptors and displays an antianxiety profile in rats but with minimal sedative effects (3–5). Some β-carboline derivatives also discriminate between binding sites in different brain regions in a fashion similar to CL-218872 (6–10). Type I receptors were suggested as those most sensitive to the carboline derivatives and CL-218872 and are most concentrated in the cerebellum. Type II receptors have a lesser sensitivity to these drugs. Although regionally varying potencies of CL-218872 suggest receptor heterogeneity, these variations could merely reflect tissue constituents (11) that vary regionally.

One of two protein bands labeled in crude brain homogenates by [^{3}H]flunitrazepam appears to be more sensitive to displacement by CL-218872 (12); however, this difference could reflect either two receptor proteins or tissue-induced modifications in mobility of a single protein. Physical separation of receptor subtypes might clarify the pharmacologic heterogeneities. Benzodiazepine receptors have been solubilized from brain membranes (13–16) and partially purified (17).

We now report differential solubilization of two benzodiazepine receptor proteins from brain membranes which show different drug specificities consistent with types I and II benzodiazepine receptors.

MATERIALS AND METHODS

Materials. [^{3}H]Flunitrazepam, [^{3}H]muscimol, and [^{3}H]propyl-β-carboline carboxylate ([^{3}H]PCC) were obtained from New England Nuclear. Unlabeled Ro22-7497 and benzodiazepines were gifts from W. Scott (Hoffmann–LaRoche). CL-218872 was obtained from Lederle Laboratories. All other chemicals were obtained from commercial sources.

Methods. Fresh calf, rat, and guinea pig brains were obtained locally and either used in experiments or frozen immediately at −70°C. Human cerebral cortex was obtained within 4 hr of death and frozen immediately. Tissues were homogenized in 10 vol of 0.05 M Tris citrate, pH 7.1/0.1 mM phenylmethylsulfonyl fluoride/1 mM Na EDTA containing 50 μg of bacitracin and 5 μg of soybean trypsin inhibitor per ml and then centrifuged at 50,000 × *g* for 15 min. Pellets were extracted in 2 vol of the homogenizing buffer containing 2% (vol/vol) Triton X-100 for 1 hr at 4°C and centrifuged at 150,000 × *g* for 1 hr. The soluble fraction was incubated with concanavalin A-Sepharose (2 ml of soluble extract per ml of packed resin) for 12–15 hr at 4°C, washed with 500 ml of 0.05 M Tris citrate, pH 7.1/0.1% Triton X-100, and resuspended to be equivalent to 4 ml per g of original tissue. The pellet was extracted a second time with 2% Triton X-100 followed by resuspension and centrifugation five times in 10 vol of 0.05 M Tris citrate (pH 7.1) and resuspended finally to 40 ml per g of original tissue. Salt extraction was performed by incubating the Triton X-100-insoluble pellet with 2 vol of the homogenizing buffer containing 2% Triton X-100 and 1 M NaCl for 1 hr at 4°C and then diluted 1:5 prior to centrifugation at 150,000 × *g* for 1 hr. Salt-soluble material was treated with concanavalin A-Sepharose as described for the Triton X-100-soluble extract. Membranes were normally prepared by washing brain homogenates 10 times by resuspension and centrifugation in 20 vol of 0.05 M Tris citrate (pH 7.1).

Membrane-bound receptor was assayed by exposure to ^{3}H-labeled ligands for 1 hr at 2°C followed by filtration and washing over Whatman GF/B filters. Soluble receptor immobilized on concanavalin A-Sepharose was assayed in a manner similar to that for membrane-bound receptor.

RESULTS

Properties of a Concanavalin Assay for Binding of ^{3}H-Labeled Ligands in Soluble Brain Fractions. To avoid the denaturation or proteolytic damage to receptor that might take place during freezing and thawing procedures used to free benzodiazepine and γ-aminobutynic acid (GABA) receptors from endogenous ligands, we adopted a novel lectin-agarose immobilization assay for soluble receptors (18).

The receptor first was bound to concanavalin A beads which were washed extensively prior to exposure to ^{3}H-labeled ligand. Soluble receptors were assayed by measuring the binding of ^{3}H-labeled ligand to the receptor immobilized on the concanavalin A beads (Table 1).

With this assay the ratio of specific to nonspecific binding to [^{3}H]flunitrazepam at benzodiazepine receptors was similar to what was obtained with a polyethylene glycol precipitation as-

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Abbreviations: GABA, γ-aminobutyric acid, Ro22-7497, methyl-β-carboline-3-carboxylate; PCC, propyl-β-carboline-3-carboxylate; CL-218872, 3-methyl-6-(3-trifluoromethylphenyl)-1,2,4-triazolo[4,3-b]pyridazine.

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Table 1. Comparison of concanavalin A (Con A)-Sepharose and polyethylene glycol (PEG) precipitation assays for solubilized benzodiazepine and GABA receptors

	Con A		PEG	
	[^{3}H]FNZ	[^{3}H]Mus	[^{3}H]FNZ	[^{3}H]Mus
Total cpm	3130	1350	2790	780
Blank cpm	150	100	190	240
Specific cpm	2980	1250	2600	540
K_d, nM	2	5	1.5	7
B_{max}, pmol/mg protein	0.1	0.3	0.18*	1*

Receptor protein from whole calf brain was solubilized with 2% Triton X-100. Some samples were treated with concanavalin A-Sepharose and washed with 100 vol of 0.05 M Tris citrate, pH 7.1/0.1% Triton X-100 prior to incubation with ^{3}H-labeled ligand for 1 hr at 2°C followed by washing over Whatman GF/B filters. Other samples were incubated directly with ^{3}H-labeled ligands and assayed by precipitation with polyethylene glycol and gamma globulin (17). Benzodiazepine and GABA receptors were labeled with [^{3}H]flunitrazepam (FNZ), and [^{3}H]muscimol (Mus), respectively (17, 19). Data are typical examples of means of triplicate samples from experiments described throughout this paper. SD < 5%.
*Values taken from ref. 13.

say. Substantially higher ratios of specific to nonspecific binding were obtained for [^{3}H]muscimol binding to GABA receptors. The maximal number of binding sites (B_{max}) and K_d value for both benzodiazepine and GABA receptors were similar with the two assays.

Differential Solubilization of Benzodiazepine Binding Sites in Various Brain Regions. Recovery of benzodiazepine and GABA receptor binding with different Triton X-100 concentrations paralleled the solubilization of total membrane protein (Fig. 1). With concentrations of Triton X-100 up to 2%, no more than 30% of benzodiazepine and 42% of GABA receptors were recovered in the soluble fraction, with corresponding losses in the membrane fractions. Two repeated reextractions of the membranes with 2% Triton X-100 failed to solubilize the remaining membrane-associated GABA and benzodiazepine receptors. After the two Triton extractions the K_d and B_{max} of benzodiazepine receptors associated with the insoluble pellet were unaltered.

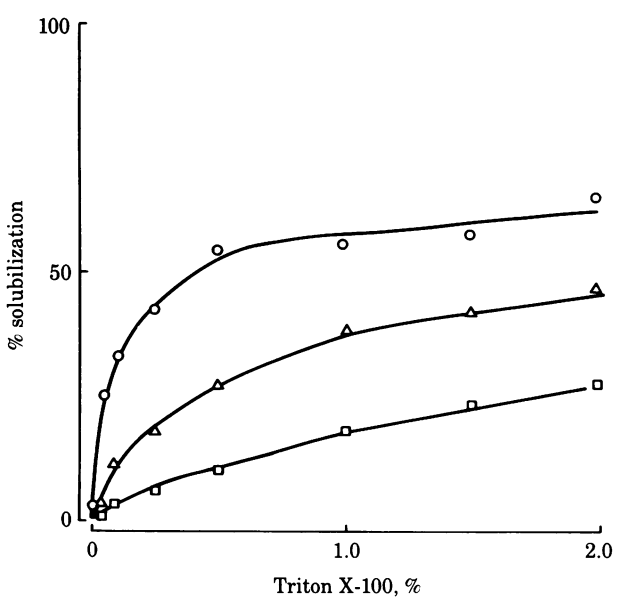

FIG. 1. Solubilization of membrane proteins and benzodiazepine and GABA receptors from whole cow brain. Membranes were extracted in Triton X-100 for 1 hr at 4°C, and the amounts of protein (○) and [^{3}H]flunitrazepam (□) and [^{3}H]muscimol (△) binding in the soluble extract and insoluble pellet were measured. The percentage solubilization was calculated as the amount of activity present in the soluble fraction divided by the total activity present in the soluble plus insoluble fractions. The experiment was repeated three times.

The degree to which benzodiazepine receptors could be solubilized from brain membranes varied with brain region (Table 2). When benzodiazepine receptor binding was measured with either [^{3}H]flunitrazepam or [^{3}H]PCC, the most extensive solubilization occurred with tissue from the brainstem and hippocampus; the least solubilization was obtained in the cerebellum and corpus striatum.

The differential solubilization of [^{3}H]flunitrazepam binding in various brain regions suggests that receptor properties vary regionally. The poor solubilization obtained in the cerebellum coincides with the great enrichment of apparent type I benzodiazepine receptor in the cerebellum (6–10) and raises the possibility that the relatively Triton X-100-insoluble benzodiazepine receptors are of the type I class.

Drug Specificity of Benzodiazepine Receptors in Triton-Soluble Fractions and Triton-Treated Membranes. To determine whether the binding sites readily solubilized by Triton X-100 are different from the binding sites that resist Triton X-100 solubilization, we compared the drug specificity of the Triton X-100-soluble fractions and the Triton X-100-treated membranes (Fig. 2). Both Ro22-7497 and CL-218872 were more potent inhibitors of [^{3}H]flunitrazepam binding in the residual Triton X-100-treated membranes than in the Triton X-100-soluble fractions; the potency in fresh membranes was intermediate. The two drugs were about 6–8 times more potent in residual membranes than in the soluble fraction from the cow brain. This same pattern of differential potencies of the two drugs occurred in different regions (cerebellum, cerebral cortex, hippocampus, and corpus striatum) of rat and cow brain and in cerebral cortex from guinea pig and human (Table 3).

^{3}H-Labeled β-carboline carboxylate derivatives are thought to have higher affinity for type I than type II benzodiazepine receptors (9, 10). [^{3}H]PCC binding to native membranes from cow or rat (Fig. 3) brain clearly showed multiphasic binding. Receptors solubilized by Triton X-100 alone showed only a low-affinity component whereas the residual pellet after Triton X-100 treatment exhibited a single class of high-affinity binding

Table 2. Ratio of soluble to insoluble benzodiazepine receptors in different regions of calf brain

	Specific [^{3}H]FNZ binding, cpm × 10^{-3} per g of original tissue		
	Soluble	Pellet	Ratio*
Corpus striatum	14.8	197.2	0.07
Cerebellum	33.7	386.8	0.09
Cerebral cortex	60.3	487.9	0.12
Midbrain	10.2	72.4	0.14
Superior colliculus	45.0	273.3	0.16
Thalamus–hypothalamus	26.8	156.1	0.17
Inferior colliculus	46.3	208.6	0.22
Brainstem	9.0	29.1	0.31
Hippocampus	59.1	131.0	0.45

Membranes prepared from different regions dissected from calf brain were solubilized in 2% Triton X-100 for 1 hr at 4°C. The amount of [^{3}H]flunitrazepam (FNZ) binding was measured in the soluble extract and in the insoluble pellet by the concanavalin A assay and membrane filtration assay, respectively. Data are the mean of two separate determinations. The values between different experiments varied less than 10%.
* Ratio is soluble/pellet.

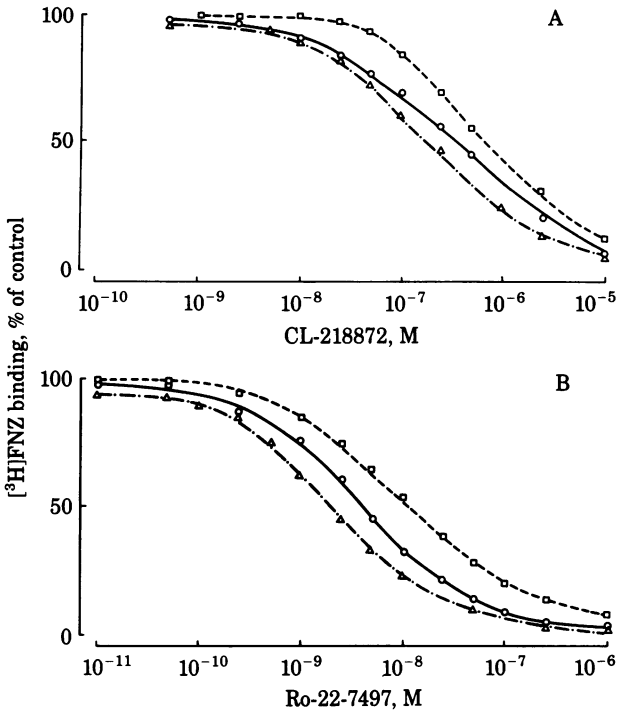

FIG. 2. Displacement of [^{3}H]flunitrazepam (FNZ) binding to benzodiazepine receptors by CL-218872 (*A*) and Ro-22-7497 (*B*). Native membranes (○) and Triton X-100-soluble (□) and Triton X-100-insoluble pellet (△) were prepared from cow cerebral cortex. Aliquots were incubated with 1 nM [^{3}H]flunitrazepam for 1 hr at 2°C in the presence of a range of concentrations of unlabeled drugs. Values shown are the means from three separate experiments.

sites. For [^{3}H]PCC binding in rat cerebral cortex, the K_d and B_{max} values are given in the legend to Fig. 3. This pattern of high-affinity binding in the residual pellet and low-affinity binding in the Triton X-100 soluble fraction also was observed in the cerebral cortex, corpus striatum, cerebellum, and hippocampus of rat or calf brain. However, the ratio of B_{max} values of [^{3}H]PCC binding for soluble and insoluble receptors varied by more than 5-fold for different regions in the order hippocampus > cerebral cortex > cerebellum > corpus striatum. Similarly, the observation that PCC has about 8–25 times greater affinity for the residual membranes after Triton X-100 treatment than for the Triton X-100-soluble fractions supports the idea that the Triton X-100-soluble and -insoluble preparations represent type II and type I receptors, respectively.

Table 3. Differential solubilization of benzodiazepine receptor binding sites with different affinities for a β-carboline ester

Species	Receptor distribution, % of native membrane		Inhibition by Ro22-7497, IC_{50}*	
	Sol.	Insol.	Sol.	Insol.
Rat	33	55	4	0.5
Cow	28	66	15	2.5
Human	8	68	4	1.0
Guinea pig	25	48	6	1.5

Cerebral cortex was extracted twice with 2% Triton X-100, and [^{3}H]flunitrazepam binding and inhibition by Ro22-7497 were assayed in the soluble extract and insoluble pellet. Data are the mean of three experiments whose results varied less than 20%. Soluble fractions (Sol.) represent binding to material dissolved by 2% Triton; insoluble (Insol.) fractions represent the residual membrane after Triton treatment.

* Concentration (nM) for 50% inhibition.

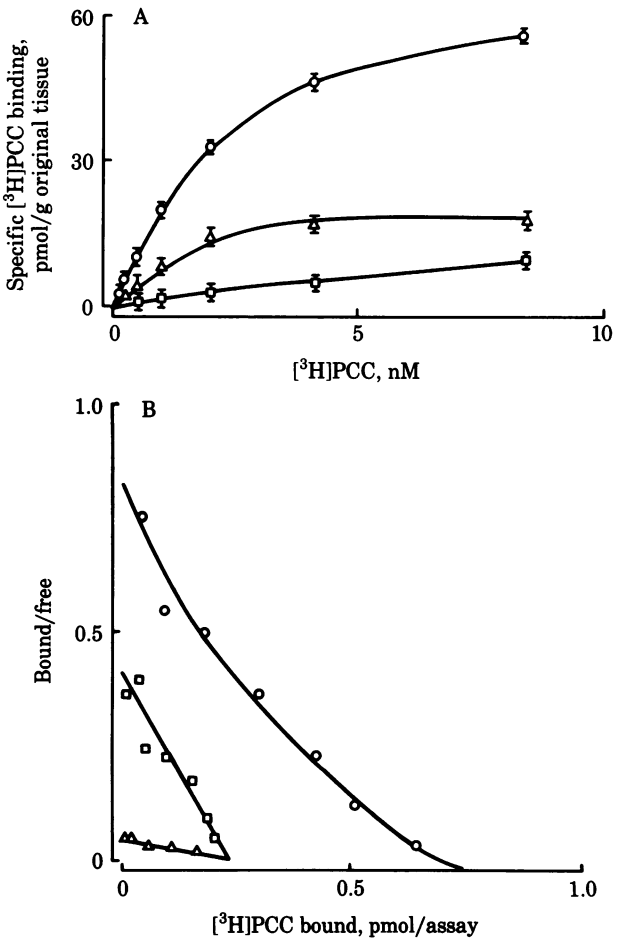

FIG. 3. [^{3}H]PCC binding to benzodiazepine receptors from rat cerebral cortex. Soluble (□) and insoluble (△) receptors were prepared from native membranes (○) by extraction with 2% Triton X-100. (*A*) Specific [^{3}H]PCC binding (mean ± SD) was determined at 2°C in 0.05 M Tris citrate (pH 7.1). (*B*) Scatchard analysis of specific [^{3}H]PCC binding. For insoluble fraction: K_d, 1.1 nM; B_{max}, 20 pmol/g. For soluble fraction: K_d, 25 nM; B_{max}, 2 pmol/g.

Recovery of Triton X-100-Insoluble Benzodiazepine Receptors by Extraction with High Salt Concentrations. To compare directly the two apparent benzodiazepine receptors, we obtained both forms in a soluble state. High salt concentrations plus detergent solubilized benzodiazepine receptors that resisted treatment with Triton X-100 alone (Table 4). When brain membranes previously extracted twice with 2% Triton X-100 were treated again with 1% or 2% Triton X-100, only about 3% of the residual benzodiazepine binding was solubilized. Similarly, treatment of these membranes with 1 M NaCl alone did not solubilize a significant portion of receptors. However, treatment of these membranes with 1 M NaCl together with 2% Triton X-100 solubilized about half of the Triton X-100-insoluble benzodiazepine receptor content. Similar results were obtained with sodium cholate together with 1 M NaCl or 1 M KSCN. A second treatment of the membranes with 1 M NaCl plus 2% Triton X-100 further solubilized about a third of the benzodiazepine sites that resisted solubilization with the initial salt plus detergent treatment.

Drug specificity experiments indicated that a major portion of the receptor solubilized by salt plus Triton X-100 treatment is of the type I class (Table 5). Thus, the potencies of Ro22-7497 and CL-218872 in the fraction solubilized by salt plus Triton X-100 are similar to those in the Triton X-100-insoluble fractions. Moreover, [^{3}H]PCC has a higher affinity for the Triton X-100-

Table 4. Solubilization of benzodiazepine receptors with Triton X-100 and Triton X-100 plus 1 M NaCl

	[^{3}]Flunitrazepam binding		
	B_{max}	Recovery, %	K_d, nM
Native membrane	32.2	100	2.0
1st Triton extract:			
Soluble	8.0	25	2.4
Insoluble	19.3	60	2.0
2nd Triton extract:			
Soluble	<1.0	3	—
Insoluble	19.0	58	—
Triton/NaCl extract:			
Soluble	9.4	29	2.1
Insoluble	9.6	30	2.2

Native membranes prepared from whole calf brain were extracted with 2% Triton X-100 for 1 hr at 4°C. The residual pellet was extracted a second time with 2% Triton X-100 alone and washed five times with 0.05 M Tris citrate (pH 7.1) by resuspension and centrifugation. The insoluble pellet was extracted with 2% Triton X-100/1 M NaCl for 1 hr at 4°C. Saturation analysis of [^{3}H]flunitrazepam binding was performed on the soluble fractions and insoluble pellets after each extraction step at 2°C. Values shown are means of three separate experiments and the variation was less than 10%. B_{max} units are pmol/g of tissue.

treated membranes and the salt plus Triton X-100-solubilized fractions than for the receptor solubilized in Triton X-100 alone.

To ensure that the differential effects of Ro22-7497 and CL-218872 on the two receptor preparations do not merely reflect the different treatment of the various fractions, we examined the influence of benzodiazepines that are thought not to differentiate between type I and type II receptors. Diazepam, clonazepam, Ro7-1986/1, and flurazepam all have the same affinities for native membranes, Triton X-100-treated membranes, Triton X-100-solubilized, and salt plus Triton X-100-solubilized fractions (Table 5). Moreover, the K_d values of [^{3}H]flunitrazepam binding for all these preparations are the same (Table 4.)

DISCUSSION

The major finding of the present study is that two types of benzodiazepine receptor can be separated physically. They appear to correspond to the type I and type II benzodiazepine receptors previously hypothesized on the basis of differential drug effects (3–5, 7–10). Evidence that the differentially detergent-solubilized receptors represent distinct receptor proteins includes the following.

Table 5. Differential drug effects on benzodiazepine receptors separated by detergent solubilization

	IC_{50}, nM*			
Drugs	Membrane	Triton sol.	Triton insol.	Triton/NaCl sol.
Ro22-7497	3	18	5	4.4
CL-218872	80	430	63	140
Diazepam	5	5	3.7	7.7
Clonazepam	6.2	6.2	5.9	5.3
Ro7-1986/1	10	15	14	19
Flurazepam	20	29	33	39

Receptor was prepared from calf cerebral cortex. The concentration of [^{3}H]flunitrazepam was 1 nM in all experiments. Values shown are the means from triplicates of two separate determinations using the concanavalin A-Sepharose assay. Two experiments using the polyethylene glycol precipitation assay showed similar values (deviation, <20%).

* Concentration for 50% inhibition.

1. The differential solubilization of the two benzodiazepine binding entities is not a continuum. Instead, progressive increases in Triton X-100 concentration and repeated extractions with Triton X-100 do not solubilize more than one-third of benzodiazepine binding sites. Moreover, the same differential solubilization has been obtained with other detergents including sodium cholate, digitonin, Nonidet P40, and Lubrol PX.

2. Of numerous drugs evaluated, differential potencies on the Triton X-100-soluble and Triton X-100-insoluble fractions are observed only with Ro22-7497 and CL-218872, which presumably differentiate type I and type II benzodiazepine receptors. Furthermore, direct binding of [^{3}H]PCC also differentiates the two benzodiazepine receptors. Several other benzodiazepine drugs have the same affinities for the two receptor preparations.

3. The differential solubilization patterns vary regionally. The density of Triton-insoluble receptors is greatest in brain areas where benzodiazepine binding has been reported to have the drug specificity of type I receptors, which coincides with our finding that the Triton X-100-insoluble binding sites display type I drug specificity.

4. The differential solubilization properties with their characteristic drug specificities are observed in brains of several species and in various regions of the brain.

5. The differential drug specificities are observed after the Triton X-100-insoluble receptors are solubilized with salt plus detergent, so the drug selectivity patterns are not merely an artifact of comparing membrane-associated and soluble receptors.

It is unclear whether the type I and type II benzodiazepine receptors that we have separated correspond in molecular weight to the two [^{3}H]flunitrazepam binding subunits observed by Sieghart and Karobath (12). Conceivably, the Triton X-100-insoluble receptors are tightly associated with cell cytoskeletal structures, which are often insoluble in nondenaturing detergents. This suggests different intracellular locations of the two receptors.

Massotti *et al.* (20) reported that as little as 0.05% Triton X-100 would solubilize up to 50% of benzodiazepine receptor sites. Under our conditions, substantially higher concentrations of detergent were required to obtain comparable degrees of receptor solubilization.

The assay for solubilized benzodiazepine and GABA receptors immobilized on concanavalin A beads may facilitate studies of soluble receptors. The immobilized receptors can be extensively washed to remove tissue constituents which might affect receptor activity. Receptor immobilization provides a partial purification (17), because only glycoproteins bind to concanavalin A. The concanavalin A assay is more rapid and involves less physical manipulation than do other assays for soluble receptors involving protein precipitation and so might be useful for detecting small ligands of relatively low affinity.

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