MOLECULAR PHARMACOLOGY, 26:458–469

Anxiolytic Cyclopyrrolones Zopiclone and Suriclone Bind to a Novel Site Linked Allosterically to Benzodiazepine Receptors

ROSARIO R. TRIFILETTI AND SOLOMON H. SNYDER

Departments of Neuroscience, Pharmacology, and Experimental Therapeutics, Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received December 30, 1983; Accepted August 28, 1984

SUMMARY

The interactions of zopiclone and suriclone, representatives of nonbenzodiazepine cyclopyrrolone anxiolytics, with central-type benzodiazepine receptors have been characterized in rat and bovine brain. While zopiclone potently (IC_{50} ~ 50 nM) inhibits [^{3}H]Ro-15-1788 binding in an apparent mass action fashion, suriclone and its metabolite 35,489 RP are extremely potent (IC_{50} ~ 350 pM and 1 nM, respectively) and display Hill coefficients of approximately 2.0. Like classical benzodiazepines, none of the cyclopyrrolones studied display selectivity for type I or type II benzodiazepine receptors. Using [^{3}H]suriclone, saturable high affinity sites for cyclopyrrolone anxiolytics were directly labeled in rat and bovine brain. The regional distribution and pharmacologic specificity of [^{3}H]suriclone and [^{3}H]Ro-15-1788 binding sites are similar, suggesting that [^{3}H]suriclone recognition sites reside on the benzodiazepine receptor complex. Unlike classical benzodiazepine agonists, such as diazepam, the binding of [^{3}H]suriclone is not modulated by GABA, Cl^-, pentobartibal, or tracazolate. Unlike those of [^{3}H]diazepam, [^{3}H]suriclone-binding sites are only minimally affected by photoaffinity labeling with flunitrazepam. Whereas the binding affinities of [^{3}H]Ro-15-1788, [^{3}H]flunitrazepam, and [^{3}H]ethyl β-carboline 3-carboxylate increase at lower temperatures, [^{3}H]suriclone binds with higher affinity at higher temperatures. Scatchard analysis of [^{3}H]flunitrazepam, [^{3}H]ethyl β-carboline 3-carboxylate, and [^{3}H]Ro-15-1788 binding in the presence of all cyclopyrrolones studied reveals an apparent noncompetitive pattern of inhibition of binding in each case; by contrast, inhibition of [^{3}H]suriclone binding by Ro-15-1788 flunitrazepam, methyl β-carboline 3-carboxylate and all of the cyclopyrrolones studied appears competitive. The dissociation kinetics of [^{3}H]Ro-15-1788 indicate that cyclopyrrolones, but not benzodiazepines, increase the dissociation rate of [^{3}H]Ro-15-1788 from its membrane receptors; the converse is true for [^{3}H]suriclone dissociation kinetics. The association kinetics of [^{3}H]suriclone suggest that suriclone induces a conformational change upon binding to receptors. Taken together, these results indicate that [^{3}H]suriclone labels a site on the benzodiazepine receptor complex allosteric to the recognition site for benzodiazepines. A model is proposed to describe the interaction between benzodiazepines and cyclopyrrolones.

INTRODUCTION

Shortly after the discovery of specific benzodiazepine-binding sites in mammalian brain (1, 2), several anxiolytic drugs lacking obvious structural similarity to classical benzodiazepines were identified which interact with benzodiazepine receptors. Examples include the triazolopyridazine CL 218,872 (3–5), the quinoline derivatives PK 8165 and PK 9084 (6), and the cyclopyrrolones zopiclone (7) and suriclone (8). While these nonbenzodiazepine anxiolytics influence brain benzodiazepine receptor binding (5–8), their precise mechanisms of action are unclear. In the present paper, we show that zopiclone and several drugs of similar structure, the anxiolytic dithiinopyrrole suriclone and its metabolites RP 35,489 and RP 46,166 influence benzodiazepine-receptor interactions allosterically at a common recognition site distinct from that of GABA,[1] chloride ion, barbiturates, and the pyrazolopyridine tracazolate. Using [^{3}H]suriclone, we have directly labeled the cyclopyrrolone recognition site and used this radioligand to explore interactions of cyclopyrrolones with brain benzodiazepine receptors.

This work was supported by United States Public Health Service Grants MH-18501, DA 00266, and NS-16375, R.S.A. Award DA-00074 to S. H. S., Training Grant GM-07309 to R. R. T., a grant from Ives Laboratory, and a grant from the McKnight Foundation.

[1] The abbreviations used are: GABA, γ-aminobutyric acid; FNZ, flunitrazepam, β-CCE, ethyl β-carboline 3-carboxylate.

0026-895X/84/060458-12$02.00/0

MATERIALS AND METHODS

Materials. [^{3}H]Diazepam (specific activity, 80 Ci/mmol), [^{3}H]3-methylclonazepam (72.4 Ci/mmol), [^{3}H]Ro-15-1788 (87.3 Ci/mmol), [^{3}H]FNZ (87.0 Ci/mmol), and [^{3}H]β-CCE (80.4 Ci/mmol) were obtained from New England Nuclear Company (Boston, MA). [^{3}H]Suriclone (85 Ci/mmol) was synthesized by Dr. Stephen Hurt at New England Nuclear. [^{3}H]Suriclone was prepared by treating the *N*-demethylated derivative of suriclone, 35,489 RP (see Fig. 1) with [^{3}H] methyl iodide. Radiochemical purity of [^{3}H]suriclone, assesed in our laboratory, exceeded 98% as judged by thin layer chromatography on Silica Gel F_{254} plates (E. Merck, Darmstadt, West Germany) in two solvent systems, acetonitrile/methanol/acetic acid (4:2:2) and acetonitrile/methanol (5:3). [^{3}H]Suriclone was stored at −80° with radiochemical purity maintained at >98% for at least 4 months.

Zopiclone, suriclone, and several of their metabolites were gifts from Dr. J. Julou, Rhone-Poulenc Recherches (Vitry-sur-Seine, France). Diazepam, flunitrazepam, clonazepam, clorazepate, Ro-15-1788, methyl β-carboline 3-carboxylate (Ro-22-7497), Ro-5-4864, and Ro-5-3663 were gifts from Dr. Peter Sorter, Hoffmann-LaRoche (Nutley, NJ). The isoquinolines PK-8165, PK-9084, and PK-11195 were supplied by Pharmindustrie Pharmuka (Gennevilliers, France). The pyrazoloquinolines CGS-8216 and CGS-9896 were from Ciba-Geigy (Ardsley, NY). Triazolam (U33,030) was supplied by Upjohn (Kalamazoo, MI). Tracazolate (ICI 136,753) was supplied by Stuart Pharmaceuticals (Wilmington, DE). All other compounds were obtained from commercial sources.

Preparation of membranes and standard radioligand-binding assays. Whole rat brains were obtained from male Sprague-Dawley rats (150–200 g) following decapitation. In some experiments, brains were dissected as noted. Tissue was homogenized in ice-cold 50 mM Tris-citrate (pH 7.2 at 21°, pH 7.6 at 0°) by Polytron (Brinkmann, Incorporated, Westbury, NY). The homogenate was centrifuged 20 min × 48,000 × *g* and the resulting pellet was washed three times further in homogenization buffer. The pellet was suspended to a final concentration of 2 mg original wet weight/ml, which was approximately 0.1 mg of protein/ml [protein determined by the method of Lowry *et al.* (9)]. Peripheral organs of the rat were dissected and crude membrane homogenates were prepared in a similar fashion as described above for brain. In other experiments where the regional distribution of binding was studied, bovine brain, obtained from a local slaughterhouse, was utilized; membranes were prepared as described for rat brain. In standard radioligand-binding assays, 750 μl of membranes prepared as described above were mixed with 100 μl of [^{3}H]suriclone, [^{3}H]Ro-15-1788, [^{3}H]β-CCE, [^{3}H]3-methylclonazepam, flunitrazepam, or [^{3}H]diazepam at the appropriate concentration and 50 μl of 50 mM Tris-citrate (pH 7.6 at 9°) buffer, which in some experiments contained an appropriate drug at the stated concentration. Nonspecific binding for [^{3}H]suriclone was defined by adding 100 μl of unlabeled suriclone, in lieu of buffer, to give a final concentration of 10 μM in the incubation medium. No significant difference in nonspecific binding of [^{3}H]suriclone was detected when 10 μM flunitrazepam, rather than 10 μM suriclone was used to define nonspecific binding. For other radioligands, nonspecific binding was defined by adding 100 μl of flunitrazepam to a final concentration of 10 μM. Mixtures were incubated 120 min at 0° (or 4, 21, 30, or 37°, in some experiments) and then filtered over Whatman GF/B filters on a cell harvester filtration manifold (model M-24R, Brandel Incorporated, Gaithersburg, MD) followed by three 3-ml washes with ice-cold Tris-citrate buffer. After addition of 8.0 ml of liquid scintillation cocktail (Formula 947, New England Nuclear Company), filters were counted in a liquid scintillation spectrometer at 46% efficiency.

Photoaffinity labeling of brain membranes with nonradiolabeled FNZ. Membranes were prepared as above and photoaffinity labeled with FNZ according to the procedure described by Hirsch (10), except that irradiation proceeded for 20 min at 12-cm distance from a long wavelength (maximal power output, 366 nm) Black-Ray B100 A UV lamp (Ultra-Violet Products, San Gabriel, CA) under constant agitation at 0°. Membranes were washed four times in 50 mM Tris-citrate buffer to remove noncovalently bound FNZ. After such treatment, negligible [^{3}H]flunitrazepam binding is detectable by filtration assay (data not shown); the membranes are thus exhaustively photoaffinity labeled with FNZ.

Dissociation kinetics experiments. For studies of dissociation kinetics of [^{3}H]suriclone or [^{3}H]Ro-15-1788, rat brain membranes (5.0 ml) were prepared as described above, but resuspended in 50 mM Tris-citrate buffer to a final concentration of 10 mg of protein/ml. [^{3}H]Suriclone (or [^{3}H]flunitrazepam) was added to a final concentration of 1.0 nM and the mixture incubated for 120 min at 0° with constant stirring. Dissociation was initiated by 100-fold dilution of membranes into 500 ml of 50 mM Tris-citrate (pH 7.6 at 0°), or the same volume of buffer containing various drugs at specified concentrations. This procedure was sufficient to prevent reassociation of [^{3}H]suriclone or [^{3}H]flunitrazepam. At the appropriate time after initiation of dissociation, triplicate 5.0-ml aliquots were collected over Whatman GF/B filters, which were washed and counted as previously described. Dissociation from nonspecific sites was monitored similarly, except that 10 μM suriclone (when [^{3}H]suriclone dissociation was studied) or 10 μM flunitrazepam (for [^{3}H] flunitrazepam) was added prior to the 120-min incubation at 0°. As control experiments revealed dissociation from nonspecific sites to be extremely rapid ($t_{1/2}$ < 1 min), in most experiments, nonspecific binding was determined only at the end of the dissociation experiment.

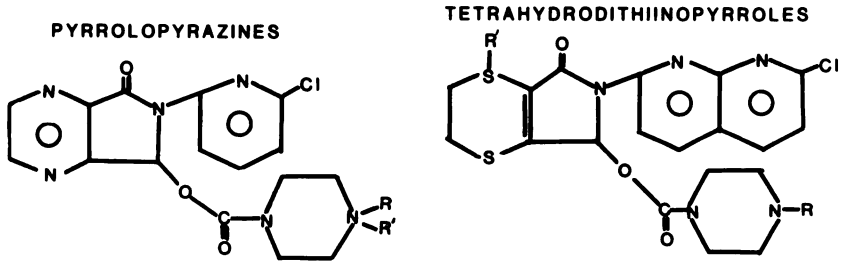
In some experiments where dissociation was initiated by addition of excess unlabeled drug, a modified procedure was followed. Membranes were prepared as described and resuspended to a protein concentration of 0.1 mg/ml in 50 mM Tris-citrate buffer. [^{3}H]Suriclone or [^{3}H] flunitrazepam were then added to a final concentration of 1 nM, and the mixture was incubated 120 min at 0°. Unlabeled suriclone or flunitrazepam (each at 10 μM final concentration) was added for [^{3}H] suriclone or [^{3}H]flunitrazepam dissociation kinetics, respectively. At various intervals after the initiation of dissociation, triplicate 1.0-ml aliquots were collected over Whatman GF/B filters and counted as above.

Association kinetics of [^{3}H]suriclone. For studies of association kinetics of [^{3}H]suriclone, 60 ml of rat brain membranes were prepared as described, and resuspended to 0.1 mg of protein/ml in 50 mM Tris-citrate buffer. [^{3}H]Suriclone was then added to the appropriate final concentration and at various times triplicate 1.0-ml aliquots were collected over Whatman GF/B filters, which were washed and counted as described above. Nonspecific association of [^{3}H]suriclone was determined for each time point in a parallel experiment in which rat brain membranes were precinbuated for 60 min with suriclone to a final concentration of 10 μM. The association of specifically bound [^{3}H] suriclone is reported.

RESULTS

Inhibition of benzodiazepine receptor binding by zopiclone, suriclone, and related agents. The structures of the cyclopyrrolone drugs evaluated are displayed in Fig. 1. The pyrrolopyrazines and dithiinopyrroles have similar structures, and both are unlike classical benzodiazepines.

Suriclone potently inhibits [^{3}H]Ro-15-1788 binding with an IC_{50} of 0.4 nM and displays a complex inhibition curve (Fig. 2). Between 0.01 and 0.1 mM, the Hill coefficient appears to be about 1.0, while at higher concentrations, the inhibition curve is steeper, with a Hill coefficient of about 2.0. RP35,489, the demethylated metabolite of suriclone, it somewhat less potent than suriclone, but shows the same pattern of inhibition. The curvilinear Hill plots for suriclone and RP35,489 appear consistently in three to five replication. RP46,166, another metabolite of suriclone, is about one-sixth as potent as suriclone itself and displays a Hill coefficient of about 1.2.



Name	R	R′
ZOPICLONE	-CH$_3$	-
29,753 RP	-CH$_3$	=O
32,273 RP	-H	-

Name	R	R′
SURICLONE	-CH$_3$	-
35,489 RP	-H	-
46,166 RP	-CH$_3$	=O

FIG. 1. *Structures of various cyclopyrrolone drugs*

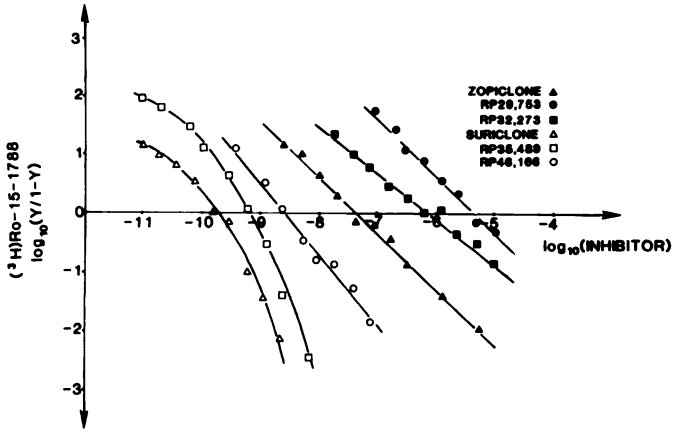


FIG. 2. *Hill plot for inhibition of [^{3}H]Ro-15-1788 binding to rat cerebral cortical membranes by various cyclopyrrolones at 0°*

Competition curves were determined as described in Materials and Methods. Data are from a representative experiment replaced two to four times. *Y* represents [^{3}H]Ro-15-1788 bound, as a fraction of control. Lines shown are weighted linear least squares fit to the experimental data. The concentration of [^{3}H]Ro-15-1788 utilized in these experiments was 0.5 nM: under these conditions, with no inhibitor added, specific binding was approximately 3,000 cpm and nonspecific binding approximately 100 cpm.

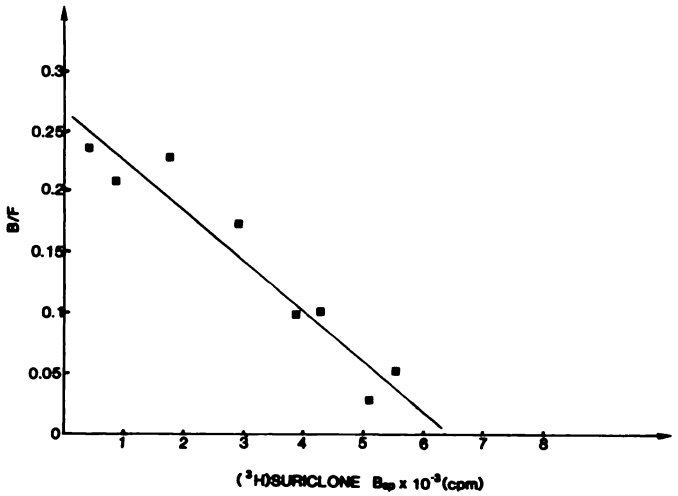


FIG. 3. *Scatchard plot analysis of [^{3}H]suriclone binding to rat cerebral cortical membranes at 0°*

Rat brain membranes were prepared and assayed for [^{3}H]suriclone binding as described in Materials and Methods. Total [^{3}H]suriclone concentration ranged from 0.05–12.5 nM in the assay. The line shown is a weighted linear least square fit through the experimental data; from this, one obtains B_{max} = 560 fmol/mg of protein and K_D = 0.29 nM. Data are from a representative experiment replicated twice.

Zopiclone is less than 1% as potent as suriclone in competing for [^{3}H]Ro-15-1788 binding; it inhibits binding with a monophasic Hill plot and Hill coefficient of 1.0. The zopiclone metabolites RP29,753 and RP32,273 are substantially less potent and show monophasic inhibition curves with respective Hill coefficients of 1.0 and 0.75.

Properties of [^{3}H]suriclone binding to rat brain membranes. As reported previously (8), [^{3}H]suriclone binds saturably and with high affinity to rat cerebral cortex membranes. In typical experiments utilizing 0.3 nM [^{3}H] suriclone at 0°, total binding is about 5,000 cpm, while nonspecific binding, measured in the presence of 10 μM suriclone, is about 500 cpm. Specific binding is saturable with half-maximal binding at about 0.25–0.3 nM and a plateau of binding at about 1 nM. Scatchard analysis (Fig. 3) indicates a single component of binding with a calculated equilibrium dissociation constant (K_D) of 290 pM and a maximal number of binding sites (B_{max}) of 560 fmol/mg of protein. Hill plot analysis (not shown) yields a Hill coefficient for the saturable specific binding of 1.0, indicating the apparent absence of homotropic cooperative interactions. [^{3}H]Suriclone binding to rat brain membranes is linear with tissue concentration up to at least 0.5 mg of protein/ml (data not shown).

Regional variations in cyclopyrrolone-receptor interactions. Considerable evidence (3–5, 11) indicates the existence of subtypes of central benzodiazepine receptors whose density varies regionally. Conceivably, the atypical influences of suriclone and related drugs on benzodiazepine binding might relate to differential effects on these receptor subtypes. Accordingly, we explored the effect of these agents on [^{3}H]Ro-15-1788 binding in rat cerebral cortex, cerebellum, and corpus striatum (Table 1), brain regions with varying putative subtype composition (12, 13). No marked differences are apparent in potencies of zopiclone, suriclone, RP 35,489, and RP 46,166 in brain membranes from these three areas. Moreover, the inhi-

TABLE 1

Regional variations in competition by cyclopyrrolones for specific [^{3}H] Ro-15-1788 binding

Competition experiments in rat brain membranes at 0° were performed as described in Materials and Methods. Each value represents the mean ± standard error with the number of separate determinations in parentheses. For suriclone and RP 35,489, Hill plots were curvilinear; in these cases, values refer to Hill coefficients using drug concentrations in the range 0.3–3 nM. Zopiclone and RP 46,166 gave monophasic Hill plots. The final concentration of [^{3}H]Ro-15-1788 in the assay was 0.5 nM in these experiments.

	IC_{50}	Pseudo-Hill coefficient
Cerebellum		
Zopiclone	45 ± 6 nM (4)	1.0 ± 0.1 (4)
Suriclone	385 ± 22 pM (4)	1.9 ± 0.2 (4)
RP 35,489	970 ± 26 pM (3)	2.0 ± 0.1 (3)
RP 46,166	1.8 ± 0.2 nM (3)	1.3 ± 0.2 (3)
Cerebral cortex		
Zopiclone	52 ± 5 nM (4)	1.06 ± 0.04 (4)
Suriclone	410 ± 20 pM (4)	2.0 ± 0.1 (4)
RP 35,489	990 ± 31 pM (3)	2.1 ± 0.2 (3)
RP 46,166	1.9 ± 0.2 nM (3)	1.1 ± 0.1 (3)
Corpus striatum		
Zopiclone	50 ± 8 nM (4)	1.02 ± 0.02 (4)
Suriclone	370 ± 35 pM (4)	2.0 ± 0.1 (4)
RP 35,489	1.03 ± 0.03 pM (3)	2.3 ± 0.2 (3)
RP 46,166	2.1 ± 0.2 nM (3)	1.12 ± 0.05 (3)

bition curves and Hill coefficients for the drugs are essentially the same in all three regions. Thus, these drugs do not appear to interact differentially with type I and type II benzodiazepine receptors.

Additionally, we have determined numbers of binding sites for [^{3}H]suriclone and [^{3}H]Ro-15-1788 in 21 different regions of bovine brain (Fig. 4). The numbers of [^{3}H]suriclone and [^{3}H]Ro-15-1788 sites are closely correlated. The average ratio of [^{3}H]suriclone to [^{3}H]Ro-15-1788 sites is 1.35 ± 0.06 in the 21 regions examined.

Both [^{3}H]Ro-15-1788 and [^{3}H]suriclone recognition sites are restricted to the central nervous system. We fail to find substantial binding of either ligand in the following peripheral organs of the rat: lung, trachea, heart, ileum, colon, epididymis, ureter, kidney, adrenal, pancreas, spleen, testis, bladder, duodenum, skeletal muscle, liver, esophagus, stomach, and vas deferens.

Pharmacologic specificity of [^{3}H]suriclone and [^{3}H]Ro-15-1788 recognition sites. We evaluated the ability of a range of drugs to compete for [^{3}H]suriclone binding to rat whole brain membranes (Table 2). The relative potencies of many drugs examined are similar in competing for [^{3}H]suriclone and [^{3}H]Ro-15-1788 binding, agreeing with the findings of others utilizing a more limited number of drugs (8). All drugs examined, other than the cyclopyrrolones zopiclone, suriclone, RP35,489, and RP46,166, are about 2–4-fold less potent in competing for [^{3}H]suriclone than [^{3}H]Ro-15-1788 binding.

The low potency of the peripheral benzodiazepine receptor-selective drugs Ro-5-4864 (14) and PK11195 (15) in competing for [^{3}H]suriclone and [^{3}H]Ro-15-1788 binding implies that neither of these drugs has high affinity for peripheral benzodiazepine receptors, which is in accord with our failure to find [^{3}H]suriclone or [^{3}H]Ro-15-1788 binding sites in peripheral organs.

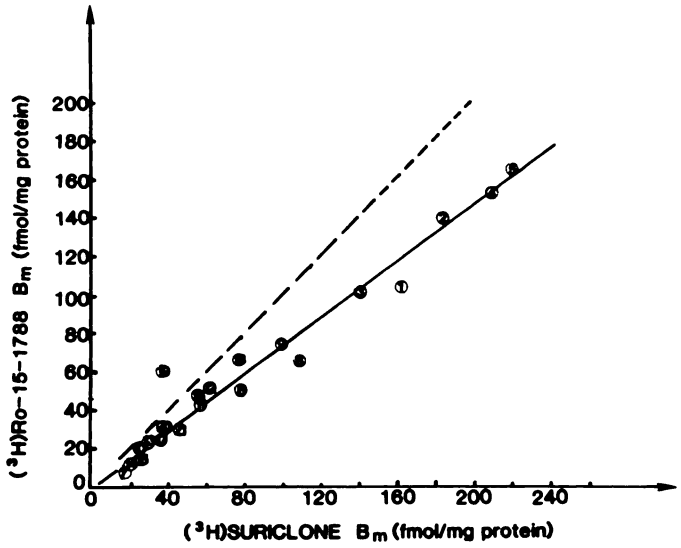


FIG. 4. *Comparative regional distribution of [^{3}H]suriclone- and [^{3}H] Ro-15-1788-binding sites to membranes prepared from various bovine brain regions*

Membranes were prepared from the following bovine brain regions according to Materials and Methods: (*1*) prefrontal cortex, (*2*) frontal cortex, (*3*) parietal cortex, (*4*) occipital cortex, (*5*) cingulate cortex, (*6*) thalamus, (*7*) pineal, (*8*) dentate gyrus, (*9*) fornix, (*10*) amygdala, (*11*) cerebellar deep nuclei, (*14*) medulla, (*15*) pons, (*16*) midbrain, (*17*) substantia nigra, (*18*) inferior colliculus, (*19*) superior colliculus, (*20*) caudate-putamen, and (*21*) globus pallidus. [^{3}H]Suriclone nd [^{3}H]Ro-15-1788 binding were assayed at a final concentration of 12.5 nM, as described in Materials and Methods; specific binding under these conditions is a measure of the B_{max} of the respective binding sites. —, weighted linear least squares fit through the experimental data; - - -, would be predicted if [^{3}H]Ro-15-1788 and [^{3}H]suriclone sites are in a 1:1 stoichiometry. Data are from a representative experiment replicated twice.

Effects of modifiers of benzodiazepine agonist binding upon [^{3}H]suriclone binding to rat cerebral cortical membranes. Zopiclone and suriclone behave pharmacologically like benzodiazepine agonists, displaying anxiolytic, anticonvulsant, and sedative-hypnotic effects in animals and humans (16–18). We examined the influence of a number of agents that selectively modulate benzodiazepine agonist binding upon the binding of [^{3}H]suriclone to rat cerebral cortical membranes (Fig. 5).

As previously reported (19), chloride ion causes a concentration-dependent stimulation (an approximately 40% increase relative to control at 300 nM) of specific binding of the benzodiazepine agonist [^{3}H]diazepam (Fig. 5A). Lesser, but still significant, stimulation of the benzodiazepine antagonist [^{3}H]Ro-15-1788 and "active antagonist" (20) [^{3}H]β-CCE occurs at 300 mM chloride. By contrast, [^{3}H]suriclone binding appears insensitive to chloride ion. As previously reported (21), GABA causes a marked, concentration-dependent stimulation of binding of the benzodiazepine agonist [^{3}H]diazepam (Fig. 5B). Binding of [^{3}H]Ro-15-1788, [^{3}H]β-CCE, and [^{3}H] suriclone is insensitive to GABA. Pentobarbital and tracazolate in the presence of 100 mM Cl^- stimulate [^{3}H] diazepam binding as previously reported (22, 23; Fig. 5C

TABLE 2

Competition for [^{3}H]suriclone and [^{3}R]Ro-15-1788 binding to rat brain membranes by various drugs

Whole rat brain membranes were prepared as described in Materials and Methods. Apparent IC_{50} values were determined using six to eight concentrations of each drug in [^{3}H]Ro-15-1788- or [^{3}H]suriclone-binding assays, respectively. In these experiments, concentrations of [^{3}H]Ro-15-1788 and [^{3}H]suriclone were 0.5 nM. Hill plots for suriclone and RP 35,489 competition for [^{3}H]Ro-15-1788 binding were curvilinear; in these cases, values refer to Hill coefficients using drug concentrations in the range 0.3–3 nM. All other drugs gave monophasic Hill plots. Data are the mean values ± standard error of the mean of three separate determinations.

Drug	Competing for [^{3}H]Ro-15-1788		Competing for [^{3}H]suriclone	
	IC_{50} nM	Hill coefficient	IC_{50} nM	Hill coefficient
CGS 9896	0.13 ± 0.02	1.02 ± 0.03	0.35 ± 0.03	1.01 ± 0.02
CGS 8216	0.22 ± 0.03	1.04 ± 0.05	1.28 ± 0.07	1.1 ± 0.2
Suriclone	0.5 ± 0.1	2.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.1
U 33,030	0.9 ± 0.1	1.03 ± 0.07	4.3 ± 0.3	1.0 ± 0.1
RP 35,489	1.0 ± 0.1	2.1 ± 0.2	0.95 ± 0.07	1.1 ± 0.1
Clonazepam	1.2 ± 0.2	0.98 ± 0.06	4.1 ± 0.2	1.03 ± 0.05
Ro-15-1788	1.2 ± 0.1	0.95 ± 0.06	8 ± 2	0.93 ± 0.06
RP 46,166	1.6 ± 0.1	1.2 ± 0.1	3.8 ± 0.5	1.05 ± 0.05
Ro-22-749Z	1.6 ± 0.2	0.82 ± 0.05	9 ± 1	0.96 ± 0.03
Flunitrazepam	3.8 ± 0.2	0.99 ± 0.04	17 ± 3	0.99 ± 0.06
Diazepam	11 ± 1	0.94 ± 0.08	64 ± 8	1.02 ± 0.04
Zopiclone	40 ± 8	1.05 ± 0.07	80 ± 10	1.0 ± 0.1
CL 218,872	160 ± 12	0.78 ± 0.06	640 ± 60	0.9 ± 0.2
PK 8165	180 ± 15	1.01 ± 0.03	1,200 ± 80	1.0 ± 0.1
Clorazepate	300 ± 20	1.04 ± 0.05	1,200 ± 60	1.1 ± 0.1
PK 9084	400 ± 40	0.96 ± 0.06	1,300 ± 90	0.93 ± 0.08
Dipyridamole	430 ± 60	0.95 ± 0.08	1,200 ± 150	0.98 ± 0.05
Ro5-4864	>10,000	ND*	>10,000	ND
PK 11195	>10,000	ND	>10,000	ND
Ro5-3663	>10,000	ND	>10,000	ND

* ND, value not determined.

and D). Neither pentobarbital nor tracazolate influences the binding of [^{3}H]Ro-15-1788, [^{3}H]β-CCE, or [^{3}H]suriclone.

Effects of photolabeling with flunitrazepam on [^{3}H] suriclone binding. Photoaffinity labeling of benzodiazepine receptors with flunitrazepam decreases the apparent receptor affinity for benzodiazepine agonists, while having negligible effects on the affinity of residual binding sites for benzodiazepine antagonists or active antagonists (10, 24, 25). Rat brain membranes were photolabeled with flunitrazepam (as indicated in Materials and Methods) for various durations and the residual binding of five radioligands monitored (Fig. 6). Photolabeling with flunitrazepam differentially affects benzodiazepine agonists (such as [^{3}H]diazepam or [^{3}H]3-methylclonazepam) and antagonists ([^{3}H]Ro-15-1788) or active antagonists ([^{3}H]β-CCE). After 30 min of irradiation, binding of both labeled benzodiazepine agonists is almost completely abolished, while binding of [^{3}H]suriclone, [^{3}H]Ro-15-1788, and [^{3}H]β-CCE is reduced 20–30%. Thus, in this paradigm, [^{3}H]suriclone behaves like the antagonist or active antagonist. No changes in binding of any of the ligands studied occur if membranes are irradiated in the absence of flunitrazepam (data not shown).

Thermodynamics of [^{3}H]suriclone binding. The binding of several benzodiazepine agonists, antagonists, and "active antagonists" to benzodiazepine receptors is temperature dependent; the receptor displays lower affinities for these drugs at 37 and 4° (26–29).

In contrast to benzodiazepine agonists, antagonists and "active antagonists," [^{3}H]suriclone displays a higher apparent potency at its recognition site at 37° than at 4 or 0° (Fig. 7). This effect of temperature involves a lowered apparent K_D, with no change in the apparent B_{max} (data not shown). As reported previously (27), the van't Hoff plot for [^{3}H]Ro-15-1788 binding (Fig. 7) is linear. The plot for [^{3}H]suriclone also appears linear and from these data one computes $\Delta H_{assoc} = -32.8$ kJ mol^{-1} and $\Delta S_{assoc} = +0.058$ kJ mol^{-1} K^{-1} for [^{3}H]Ro-15-1788 and $\Delta H_{assoc} = +46.0$ kJ mol^{-1}, $\Delta S_{assoc} = +0.350$ kJ mol^{-1} °K^{-1} for [^{3}H]suriclone. Thus, the binding of [^{3}H]Ro-15-1788 is enthalpically driven over the range 0–37°, while the binding of [^{3}H]suriclone is entropically driven over this temperature range.

Influence of zopiclone, suriclone, and related agents on benzodiazepine receptor and [^{3}H]suriclone-binding saturation. We explored the influence of zopiclone, suriclone, and related agents upon the equilibrium binding properties of [^{3}H]Ro-15-1788 (Fig. 8). Zopiclone and suriclone decrease B_{max} values with no significant effects on the K_D for [^{3}H]Ro-15-1788. The suriclone metabolites RP35,489 and RP46,166 give similar results (data not shown). All of these drugs also decrease the B_{max} without altering the K_D for [^{3}H]β-CCE and [^{3}H]flunitrazepam binding (data not shown). By contrast benzodiazepines, such as diazepam and flunitrazepam, increase the K_D for these three radioligands with no change in B_{max} (data not shown).

We also examined the saturation of [^{3}H]suriclone binding to rat brain membranes in the presence of various benzodiazepine and cyclopyrrolone drugs, the converse of the experiment described above. Ro-15-1788,

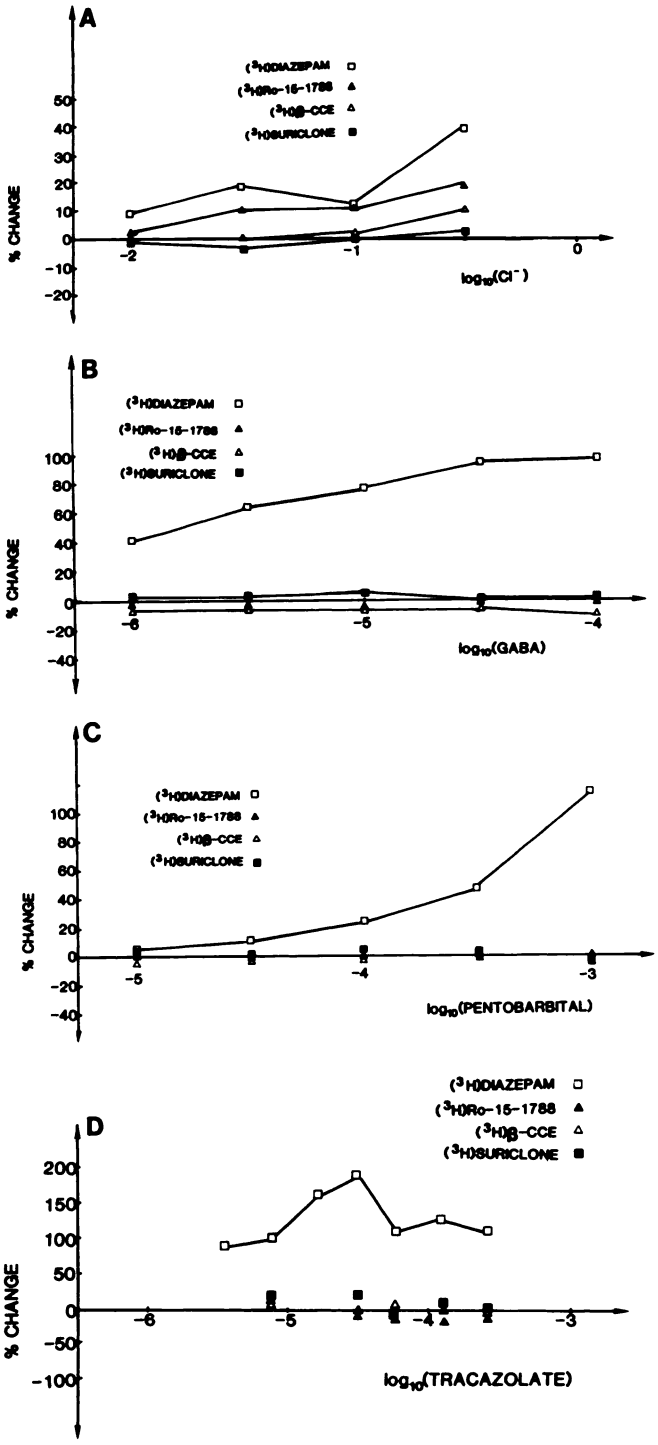


FIG. 5. *Effects of modifiers of benzodiazepine agonist binding upon [^{3}H]diazepam, [^{3}H]Ro-15-1788, [^{3}H]β-CCE, and [^{3}H]suriclone binding to rat cerebral cortical membranes at 0°*

Membranes were prepared and assayed with either 5.0 nM [^{3}H]diazepam, 0.5 nM [^{3}H]Ro-15-1788, 0.5 nM [^{3}H]β-CCE, or 0.5 nM [^{3}H]suriclone. Data are expressed as the per cent change in specific binding relative to control binding in the absence of modifier. Data are presented for (A) chloride ion, (B) GABA, (C) pentobarbital, and (D) tracazolate. For the latter two potential modifiers, Cl^- was added at a concentration of 0.1 M, as the ability of these agents to influence benzodiazepine agonist binding is chloride ion-dependent. Data are from a representative experiment replicated twice.

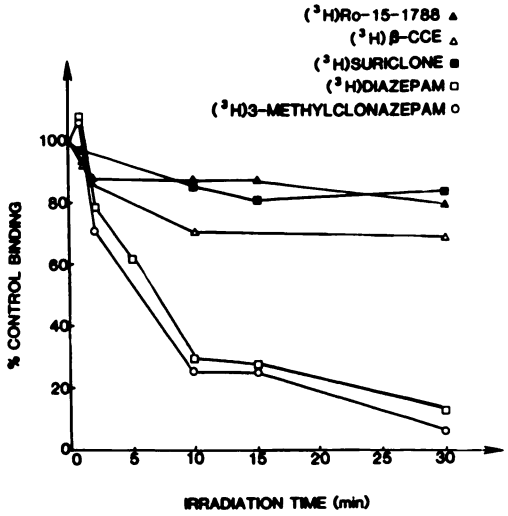


FIG. 6. *Effect of photoaffinity labeling of rat cerebral cortical membranes with nonradiolabeled flunitrazepam upon the binding of [^{3}H]Ro-15-1788, [^{3}H]suriclone, [^{3}H]β-CCE, [^{3}H]diazepam, and [^{3}H]3-methyl clonazepam binding to rat cerebral cortical membranes at 0°*

Rat cerebral cortical membranes were prepared and photolabeled with flunitrazepam for various irradiation times as described in Materials and Methods. After extensive washing, membranes were assayed with [^{3}H]Ro-15-1788, [^{3}H]suriclone, [^{3}H]β-CCE, [^{3}H]methylclonazepam (each at a final concentration of 0.5 nM), or [^{3}H]diazepam at a final concentration of 5.0 nM, as described in Materials and Methods. Data are presented as a fraction of the total binding present in membranes which were not irradiated, but otherwise treated identically to irradiated membranes. Typical control specific binding for [^{3}H]methylclonazepam was approximately 2000 cpm and nonspecific binding 200 cpm; values for other radioligands are as indicated in the legend to Fig. 5. Data are from a representative experiment replicated twice.

flunitrazepam, methyl β-carboline 3-carboxylate and zopiclone all behave as apparent competitive inhibitors of [^{3}H]suriclone binding (Fig. 9). Suriclone, RP35,489, and RP46,166 also inhibit [^{3}H]suriclone binding in an apparently competitive fashion. Thus, the pattern of inhibition of benzodiazepine binding by cyclopyrrolones appears different than the pattern of inhibition of cyclopyrrolone binding by benzodiazepines.

Effects of zopiclone and suriclone on the dissociation of [^{3}H]flunitrazepam and [^{3}H]suriclone. The complex interactions of zopiclone and suriclone with benzodiazepine-binding sites may be allosterically mediated. Accordingly, one might expect zopiclone and suriclone to alter the dissociation of radiolabeled benzodiazepines from receptor sites. We evaluated the dissociation of [^{3}H]flunitrazepam from bovine cerebral cortex membranes with dissociation initiated by limiting dilution, as described in Materials and Methods, in the absence or the presence of 1 μM zopiclone, suriclone, or diazepam (Fig. 10A). In the absence of added drugs, the dissociation of [^{3}H]flunitrazepam is biphasic, as others have found (30). Both zopiclone and suriclone markedly accelerate the dissociation of [^{3}H]flunitrazepam. When dissociation of [^{3}H]flunitrazepam is initiated by 10 μM flunitrazepam, 10 μM zopiclone, or 10 μM suriclone (without dilution of membranes), results quite similar to the corresponding results obtained with limiting dilution are observed (Fig. 10B).

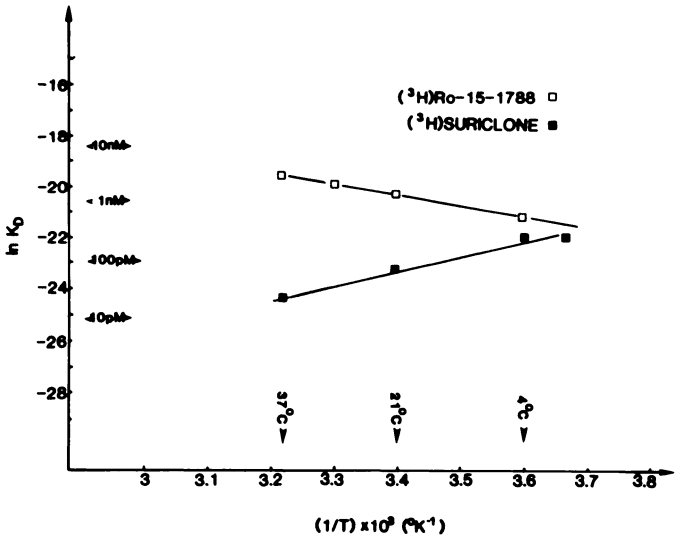


FIG. 7. *van't Hoff plot for [³H]suriclone and [³H]Ro-15-1788 binding to rat cerebral cortical membranes*

Rat cerebral cortical membranes were prepared and assayed as described in Materials and Methods at various temperatures. The value of K_D for [³H]suriclone and [³H]Ro-15-1788 was determined at each temperature and these data were transformed to a van't Hoff plot depicted above. Lines drawn are weighted least squares fit to the experimental data. Data are from a representative experiment replicated twice.

We also explored the influences of various drugs on the dissociation of [³H]suriclone from its receptor sites (Fig. 10C). When dissociation of [³H]suriclone from receptors is initiated by infinite dilution into buffer, dissociation proceeds monophasically; the calculated k_{-1} is 5.3×10^{-5} sec^{-1}, similar to what others have found (8). Dissociation by limiting dilution into 10 μM zopiclone (or 10 μM suriclone) produces similar results as limiting dilution into buffer. By contrast, limiting dilution into 10 μM flunitrazepam produces near doubling of the dissociation rate of [³H]suriclone; under these conditions, $k_{-1} = 9.1 \times 10^{-5}$ sec^{-1}. When dissociation of [³H]suriclone is initiated by 10 μM zopiclone, 10 μM suriclone, or 10 μM flunitrazepam (without dilution of membranes), results are quite similar to those obtained with limiting dilution. Thus, benzodiazepines alter the dissociation kinetics of cyclopyrrolones and cyclopyrrolones alter the dissociation kinetics of benzodiazepines.

Association kinetics of [³H]suriclone. Quast and Mahlmann (31) proved evidence for a flunitrazepam-induced conformational change by a study of [³H]flunitrazepam association kinetics at a variety of concentrations of labeled ligand. In a similar fashion, we explored the association kinetics of [³H]suriclone at several [³H]suriclone concentrations (Fig. 11), under pseudo-first order conditions. The association of [³H]suriclone to receptor is monophasic at all ligand concentrations examined (Fig. 11A). A detailed analysis of the ligand concentration dependence of [³H]suriclone association rate is presented in the Appendix. This analysis indicates that [³H]suriclone induces a conformational change upon binding to receptor.

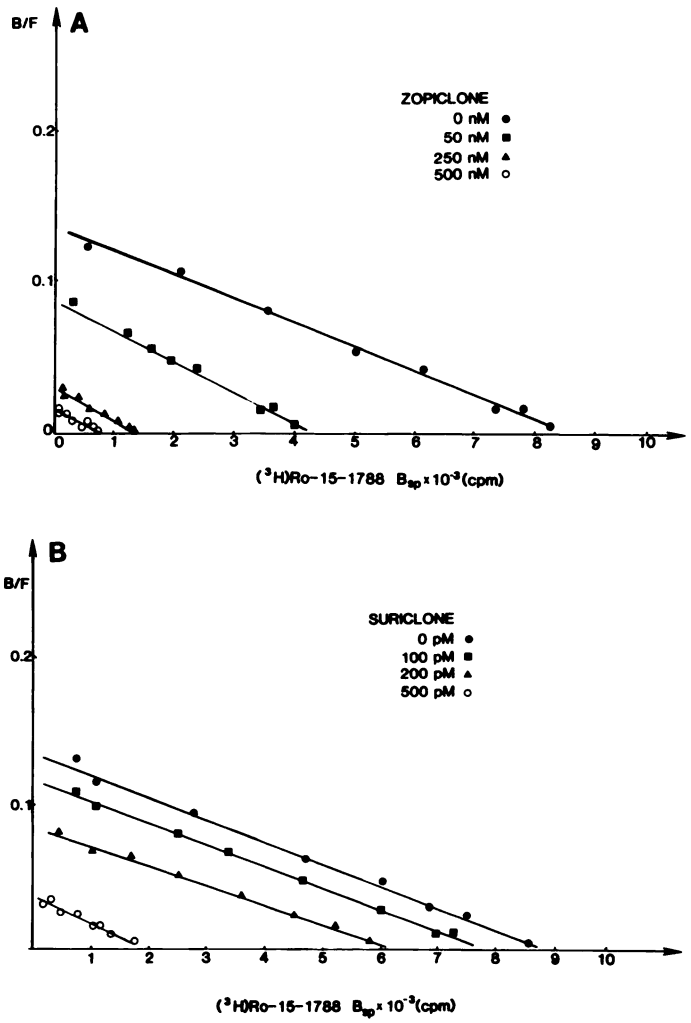


FIG. 8. *Scatchard plot of [³H]Ro-15-1788 binding to rat cerebral cortical membranes in the presence of various cyclopyrrolones at 0°*

Rat cerebral cortical membranes were prepared and saturation analyses performed as described in Materials and Methods. Results were obtained in the presence of indicated concentrations of (A) zopiclone and (B) suriclone. The final [³H]Ro-15-1788 concentration utilized in these experiments ranged from 0.05–50 nM. Data are from a representative experiment replicated twice. Lines shown are the weighted linear least squares fit to the experimental data.

DISCUSSION

The major finding of the present study is that zopiclone, suriclone, and certain of their metabolites influence benzodiazepine receptor binding in an allosteric fashion. Evidence against a simple competitive interaction includes: 1) all of the cyclopyrrolones studied reduce the apparent B_{max} of [³H]Ro-15-1788, [³H]β-CCE, and [³H]FNZ binding with no significant effect upon K_D; and 2) cyclopyrrolones, but not benzodiazepines, accelerate the dissociation of [³H]flunitrazepam from its membrane receptors and benzodiazepines accelerate [³H]suriclone dissociation.

Unlike benzodiazepine agonists, [³H]suriclone binding is not modulated by GABA, Cl^-, pentobarbital, or tracazolate, nor is it markedly reduced by photoaffinity labeling with unlabeled FNZ. Both increased affinity for benzodiazepine receptors in the presence of 100 μM

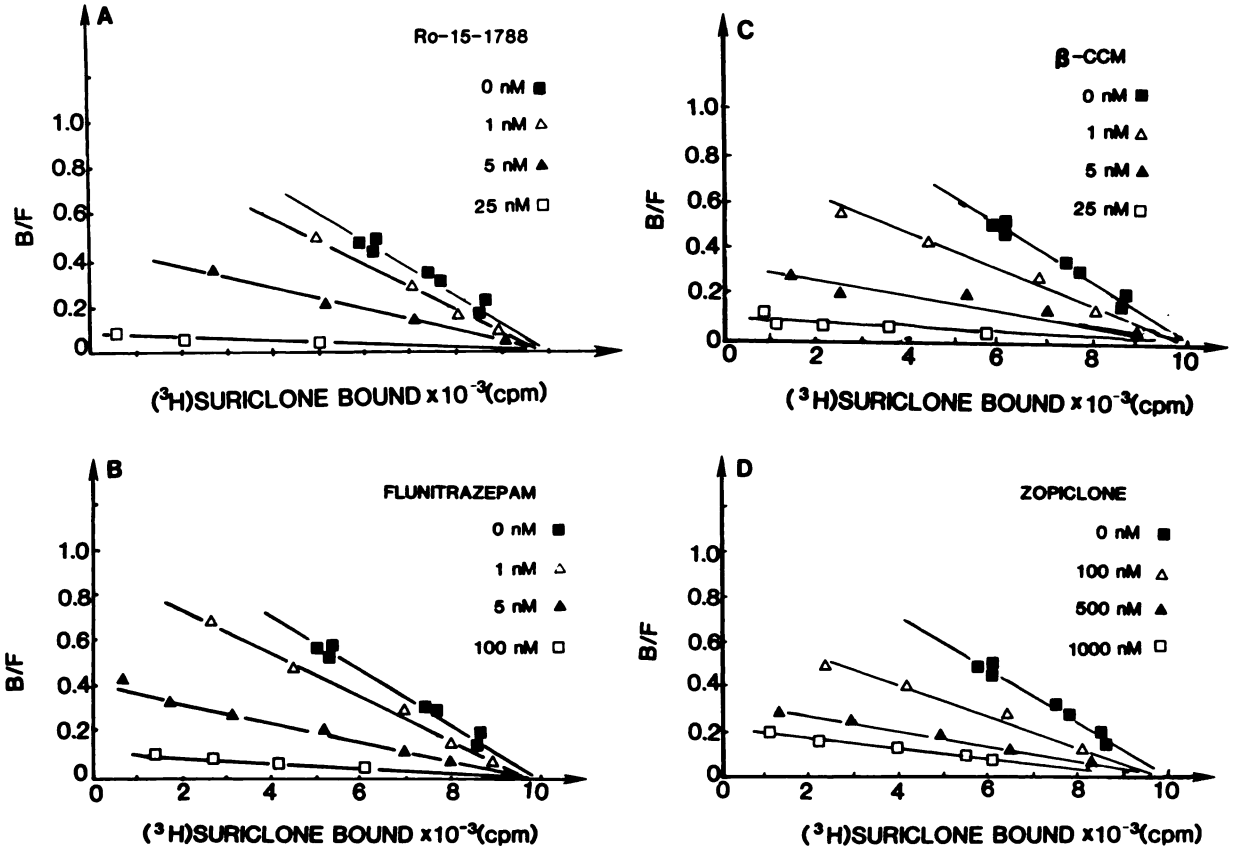


FIG. 9. *Scatchard analysis of [³H]suriclone binding to rat cerebral cortical membranes at 0° in the presence of a series of concentrations of various drugs*

Rat cerebral cortical membranes were prepared as described in Materials and Methods. A, results in the presence of 0 nM, 1 nM, 5 nM and 25 nM Ro-15-1788. B, results in the presence of 0 nM, 2 nM, 5 nM, and 100 nM flunitrazepam. C, results in the presence of 0 nM, 1 nM, 5 nM, and 25 nM methyl β-carboline 3-carboxylate. D, results in the presence of 0 nM, 100 nM, 500 nM, and 1 μM zopiclone. The final total [³H]suriclone concentration utilized in these experiments ranged from 0.05–10 nM. Data are from a representative experiment replicated twice. Lines shown are weighted linear least squares fit to the experimental data.

GABA and decreased affinity after exhaustive FNZ photoaffinity labeling are criteria which have been proposed (32) for placing anxiolytics on an agonist/antagonist continuum based on *in vitro* radioligand binding. Since cyclopyrrolones behave in intact animals and humans as pure benzodiazepine agonists (16–18), one must be cautious in using these criteria to place nonbenzodiazepine drugs (such as suriclone and zopiclone) on an agonist/antagonist continuum.

The thermodynamics of [³H]suriclone-receptor interactions differs from those of [³H]flunitrazepam (28, 29), [³H]Ro-15-1788 (27), or [³H]β-CCE (26), further supporting the proposal that cyclopyrrolones interact with a unique site. [³H]Suriclone is 10 times more potent at 37° than at 0°: at 37°, it is almost 100-fold more potent than [³H]Ro-15-1788. The extremely high potency (approximately 30 pM) of [³H]suriclone at 37° suggests that [³H]suriclone will be useful for labeling benzodiazepine receptors *in vivo*. The binding of [³H]suriclone is entropically driven, and so major contributors to its binding might be the release of ordered water or the creation of new internal degrees of freedom in the receptor molecule as a consequence of binding.

[³H]Suriclone association kinetics suggest that suriclone induces a conformational change in its receptor upon binding as proposed for flunitrazepam (31). We propose a model (Fig. 12) for the interaction of suriclone and flunitrazepam which accounts for many of the features described here. It is assumed that flunitrazepam (L) and suriclone (Z) recognize a similar receptor complex R and bind to it at distinct sites to form reversible complexes RL and RZ, respectively, in a rapid fashion. Either complex can undergo a conformational change to give the stable complexes $R'L$ and $R''Z$, respectively; R' and R'' are assumed to be distinct conformations. In the presence of [³H]suriclone, we have provided evidence that >98% of receptor is in the $R''Z$ form (see Appendix); others have suggested that ~98% of the receptor is in the $R'L$ form in the presence of [³H]flunitrazepam (31); thus, suriclone "drives" the receptor toward the R'' conformation and flunitrazepam drives the receptor toward the R' conformation. Using the rate constants for [³H]flunitrazepam binding reported by Quast and Mahlmann (31) and those for [³H]suriclone obtained here, we can see that the suriclone-receptor terminal complex, $R''Z$, reverses to form the precomplex, RZ, about 20 times slower than the corresponding reaction for flunitrazepam, i.e., $k_{-2}/k_{-1} \sim 0.05$, in the model of Fig. 12.

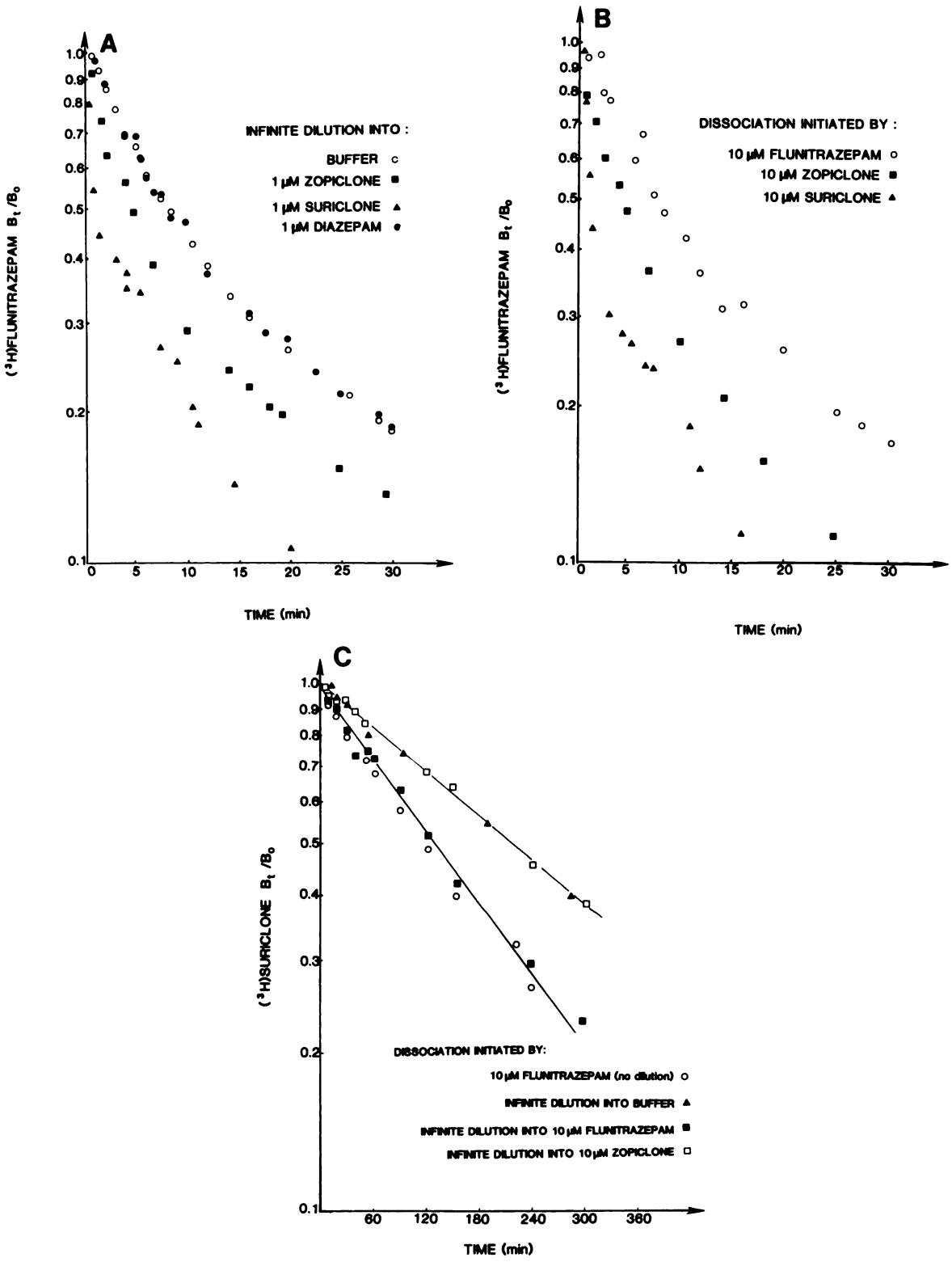


FIG. 10. *Dissociation from rat cerebral cortical membranes at 0°*

A, dissociation of [^{3}H] flunitrazepam initiated by limiting dilution into various media. Rat cerebral cortical membranes were prepared and dissociation kinetics determined as described in Materials and Methods. Dissociation was initiated by 100-fold dilution into 50 nM Tris-citrate (pH 7.2) buffer or the same buffer containing 1 μM zopiclone, 1 μM suriclone or 1 μM diazepam. Total specific binding prior to initiation of dissociation was approximately 3500 cpm and nonspecific binding was 600 cpm. Data are from a representative experiment replicated two times. B, dissociation of [^{3}H]flunitrazepam from rat cerebral cortical membranes at 0° initiated by addition of 10 μM flunitrazepam, 10 μM zopiclone, or 10 μM suriclone. Binding prior to initiation of dissociation was as described in A. Dissociation was determined as described in Materials and Methods. Results are from a representative experiment replicated twice. C, dissociation of [^{3}H]suriclone from rat cerebral cortical membranes at 0° initiated by various procedures. [^{3}H]Suriclone dissociation kinetics were determined as described in Materials and Methods. Total specific binding prior to initiation of dissociation was approximately 7000 cpm and nonspecific binding was 1000 cpm. Results are from a representative experiment replicated twice. All lines drawn are weighted linear least squares fit to the experimental data. Note that the ordinates of A–C are logarithmic scales.

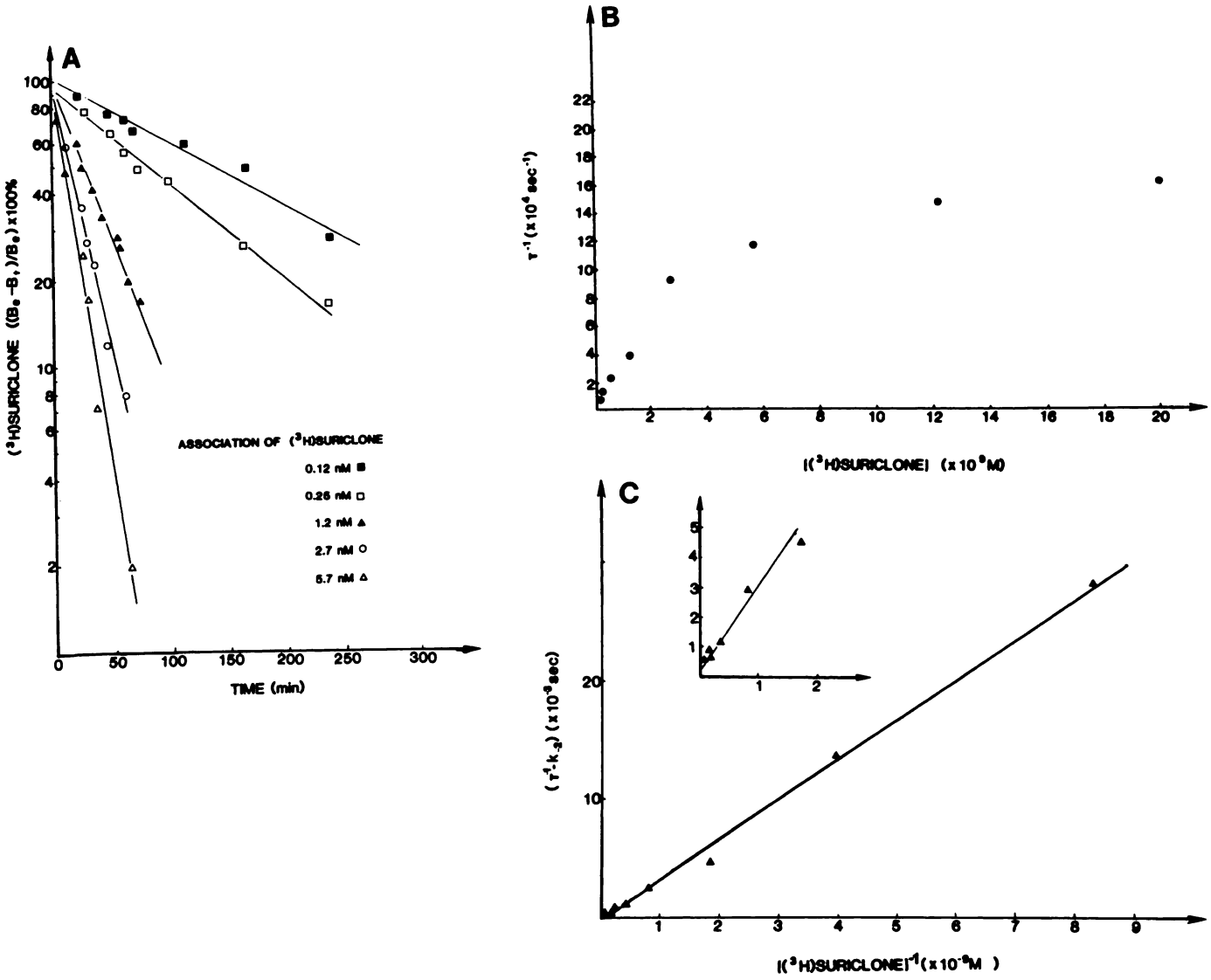


FIG. 11. *Association of [³H]suriclone to rat cerebral cortical membranes*

Rat cerebral cortical membranes were prepared, and association kinetics determined as described in Materials and Methods. A, Association of [³H]suriclone at 0.12 nM, 0.25 nM, 1.2 nM, 2.7 nM, and 5.7 nM [³H]suriclone under pseudo-first order conditions. For clarity, data for 0.81, 0.54, 0.21 nM [³H]suriclone are not shown. Lines drawn are the weighted linear least squares fit of the experimental data. Data are from a representative experiment replicated twice. Note that the ordinate is a logarithmic scale. B, dependence of association reaction rate (τ^{-1}) for [³H]suriclone binding upon [³H]suriclone concentration. The relevant calculations from the results of Fig. 11A required to construct this plot are discussed in the Appendix. C, plot of $(\tau^{-1} - k_{-2})^{-1}$ versus reciprocal [³H]suriclone concentration. The rationale behind this plot is discussed in the Appendix. —, weighted linear least squares fit of the experimental data. Inset: detail of plot near the origin.

This means that flunitrazepam drives the receptor into a conformation that is readily reversible, but suriclone drives the receptor into a conformation that is slowly reversible. To account for the effects of suriclone on the dissociation rate of [³H]flunitrazepam observed here, we assume that the ternary complex $R'LZ$ can form; to explain the reciprocal effects of flunitrazepam on [³H]suriclone binding, we assume that the ternary complex $R''LZ$ forms. Both ternary complexes relax more rapidly than the terminal complexes ($R'L$ and $R''Z$) to the precomplex forms (RL and RZ).

The proposed model can account for most of the observed features of the interaction between benzodiazepines and cyclopyrrolones.

1) As cyclopyrrolones and benzodiazepines bind to different sites on the same receptor complex, perhaps (but not necessarily) on the same protein, [³H]suriclone and [³H]Ro-15-1788 binding sites have similar regional localizations. These sites need not be in 1:1 stoichiometry, however; this might account for our observed ratio of B_{max} of [³H]suriclone sites to [³H]Ro-15-1788 sites of 1.35 ± 0.06.

2) RL and RZ are in equilibrium with the same receptor R. As the isomerizations to the stable complexes $R'L$ and $R''Z$ are rapid, the ternary complex RLZ does not form in appreciable amounts in the time scale of receptor-binding experiments. Therefore, the pharmacologic specificity of benzodiazepine and cyclopyrrolone recog-

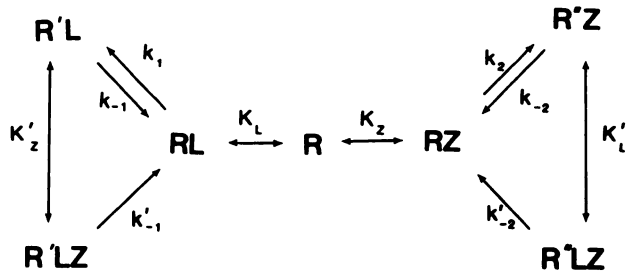


FIG. 12. *A hypothetical model for the interaction of benzodiazepines (L) and cyclopyrrolones (Z) at benzodiazepine receptors*

The model is discussed in detail in the text. For the particular case L = flunitrazepam, Z = suriclone, eight of the 10 parameters can be estimated using data of this paper, and ref. 31; K_L = 24.8 nM (ref. 31), K_Z = 11.0 nM (this paper), k_{-1} = 9 × 10^{-4} sec^{-1} (ref. 31), k_1 = 2.8 × 10^{-2} sec^{-1} (ref. 31), k'_1 = 5 × 10^{-3} sec^{-1} (this paper), k_2 = 3.3 × 10^{-3} sec^{-1} (this paper), k_{-2} = 5 × 10^{-5} sec^{-1} (this paper), and k'_2 = 9 × 10^{-6} sec^{-1} (this paper).

nition sites can be very similar. To account for the observed competition of cyclopyrrolone binding by benzodiazepines and vice versa, as well as the kinetic effects of cyclopyrrolones and benzodiazepines on one another, we must assume L has a much lower affinity for $R''Z$ than R and Z has a much lower affinity for $R'L$ than R.

3) The inhibition of radiolabeled benzodiazepine binding by cyclopyrrolones appears to be noncompetitive, because the cyclopyrrolones drive the receptor into the $R''Z$ state which reverses only slowly (in the time scale of radioligand-binding experiments). Practical considerations preclude labeling with sufficient L to form appreciable amounts of the $R''LZ$ complex, which would relax more rapidly to RZ. By contrast, the presence of high concentrations of labeled Z can overcome inhibition by L, as the $R'L$ complex is in more rapid equilibrium with its precomplex form; therefore, the inhibiton of radiolabeled cyclopyrrolone binding by benzodiazepines appears competitive.

4) As R' and R'' are different conformations of the same receptor, it is possible to understand how R' might be regulated by Cl^-, GABA, pentobarbital and tracazolate, whereas R'' is relatively insensitive to these agents (here R' specifically refers to the conformation induced by a benzodiazepine agonist, such as [^{3}H]flunitrazepam).

5) Since R' and R'' are different conformations, it is more plausible that L and Z might display different thermodynamics of binding to receptor. L might drive receptor into a relatively "tight" form R', which can stabilize the binding of L in an exothermic fashion, while the binding of Z might be driven by its ability to drive receptor into the "loose" form R''.

The model in Fig. 12 can be made quantitative for the flunitrazepam-suriclone receptor interaction, using data from Quast and Mahlmann (31) and this paper. Eight of the 10 parameters of the model can be estimated from the available data with only two, K_L' and K_Z', the stability constants of the ternary complexes, being difficult to measure.

ACKNOWLEDGMENTS

We thank Robert O. Blaustein and Adele M. Snowman for their excellent technical assistance and Dawn C. Dodson for secretarial assistance.

APPENDIX

As discussed elsewhere (31), for a biomolecular association mechanism

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RL \qquad (1)$$

one should find

$$\frac{B_e - B_t}{B_e} = \exp\left(-\frac{t}{\tau}\right) \qquad (2)$$

with

$$\tau^{-1} = k_1(L)_0 + k_{-1} \qquad (3)$$

where B_e and B_t are the specific binding of ligand at equilibrium and time t, respectively, when the association is performed at a free ligand concentration $(L)_0$. The parameter τ, termed the relaxation time, is the time required to associate to $(1 - e^{-1}) \times 100\%$ or 63.2% of the equilibrium value. At this time, $(B_e - B_t)/B_e = 0.368$, and the value of τ may be directly read from Fig. 11A. According to Eq. 3, τ^{-1} ought to be linearly related to (L_0) if the association of suriclone with receptor follows the simple bimolecular mechanism, Eq. 1. A plot of τ^{-1} versus (L_0) for [^{3}H]suriclone association is shown in Fig. 11B. As in the case of [^{3}H]flunitrazepam (31), τ^{-1} is indeed linearly dependent upon [^{3}H]suriclone concentration of low radioligand concentrations (less than about 4 nM); at higher concentrations, τ^{-1} appear to plateau. At low [^{3}H]suriclone concentrations, Eq. 3 appears to apply, so that one can determine K_{-1} and K_1 as the ordinate intercept and limiting slope of the plot in Fig. 11B. In this way, we find k_{-1} = 5 × 10^{-5} sec^{-1} and k_1 = 2.3 × 10^5 M^{-1} sec^{-1}, so that (K_D)app = k_{-1}/k_1 = 0.22 nM. These values are in good agreement with the values determined independently from dissociation kinetic and radioligand binding studies, respectively.

As Quast and Mahlmann (31) have emphasized, a simple way to account for a curvilinear plot of τ^{-1} versus $[L]$ is to propose precomplex formation followed by a ligand-induced conformational change to a final complex.

$$R + L \overset{K_L}{\rightleftarrows} RL \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} R'L \qquad (4)$$

where K_L is a *dissociation* constant and the second step is rate determining. This mechanism is assumed applicable for *all* ligand concentrations.

As others have shown (31), for the mechanism depicted in Eq. 4,

$$\tau^{-1} = k_{-2} + k_2\left(\frac{L_0}{L_0 + K_L}\right) \qquad (5)$$

Eq. 5 can be rearranged to

$$\frac{1}{\tau^{-1} - k_{-2}} = \frac{1}{k_2}\left(\frac{L_0 + K_L}{L_0}\right) = \frac{1}{k_2}\left(1 + \frac{K_L}{L_0}\right) \qquad (6)$$

As k_{-2} may be obtained as the limiting ordinate intercept of the plot of Fig. 11B, it is possible to plot $(\tau^{-1} - k_{-2})^{-1}$ versus $(L)_0^{-1}$. Eq. 6 shows such a plot ought to be linear with ordinate intercept k_2^{-1} and slope K_L/k_2 is mechanism (4) holds. When such a plot is constructed (Fig. 11c), one finds that it is indeed linear over the entire concentration range examined. From the plot of Fig. 11C, one can compute $k_2 = 3.3 \times 10^{-3}$ sec^{-1} and $K_L/k_2 = 3.33 \times 10^{-6}$ sec^{-1}, so that $K_L = 11$ nM. We compute $K_2 = k_{-2}/k_2 = 0.015$, implying that at equilibrium $(R'L)/(RL) \sim 67$. In equilibrium radioligand binding experiments, we observe the net reaction

$$R + L \overset{(K_D)\text{app}}{\rightleftharpoons} R'L \qquad (7)$$

for which $(K_D)\text{app} = K_L K_2 = 0.17$ nM, agreeing well with values determined from equilibrium binding studies. These results suggest that suriclone induces a conformational change upon binding to the benzodiazepine receptor.

REFERENCES

1. Squires, R. F., and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* **266**:732–734 (1977).
2. Mohler, H., and T. Okada. Benzodiazepine receptor: demonstration in the central nervous system. *Science* **198**:849–851 (1977).
3. Lippa, A. S., J. Coupet, E. N. Greenblatt, C. A. Klepner, and B. Beer. A synthetic nonbenzodiazepine ligand for benzodiazepine receptors: a probe for investigating neuronal substrates of anxiety. *Pharmacol. Biochem. Behav.* **11**:99–106 (1979).
4. Klepner, C. A., A. S. Lippa, D. I. Benson, M. C. Sano, and B. Beer. Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmacol. Biochem. Behav.* **11**:457–462 (1979).
5. Niehoff, D. L., R. O. Mashal, W. D. Horst, R. O'Brien, J. M. Palacios, and M. J. Kuhar. Binding of a radiolabeled triazolopyradazine to a subtype of benzodiazepine receptor in the rat cerebellum. *J. Pharmacol. Exp. Ther.* **221**:670–675 (1982).
6. LeFur, G., J. Mizoule, M. C. Burgeuin, O. Ferris, M. Heaulme, A. Gaultier, C. Gueremy, and A. Uzan. Multiple benzodiazepine receptors: evidence of dissociation between anti-conflict and anti-convulsant properties by PK 8165 and PK 9084 (two quinoline derivatives). *Life Sci.* **28**:1439–1448 (1981).
7. Blanchard, J. C., A. Boireau, C. Garret, and L. Julou. *In vitro* and *in vivo* inhibition by zopiclone of benzodiazepine binding to rodent brain receptors. *Life Sci.* **24**:2417–2420 (1979).
8. Blanchard, J. C., and L. Julou. Suriclone: a new cyclopyrrolone derivative recognizing receptors labelled by benzodiazepines in rat hippocampus and cerebellum. *J. Neurochem.* **40**:601–607 (1983).
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
10. Hirsch, J. D. Photolabeling of benzodiazepine receptors spares [^{3}H]propyl β-carboline binding. *Pharmacol. Biochem. Behav.* **16**:245–248 (1982).
11. Lippa, A. S., L. R. Meyerson, and B. Beer. Molecular substrates of anxiety: clues from the heterogeneity of benzodiazepine receptors. *Life Sci.* **31**:1409–1417 (1982).
12. Young, W. S., III, D. Niehoff, M. J. Kuhar, B. Beer, and A. S. Lippa. Multiple benzodiazepine receptor localization by light microscopic radiohistochemistry. *J. Pharmacol. Exp. Ther.* **216**:425–430 (1981).
13. Asano, T., Y. Yamada, and N. Ogasawara. Characterization of solubilized GABA and benzodiazepine receptors from various regions of bovine brain. *J. Neurochem.* **40**:209–214 (1983).
14. Braestrup, C., and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high affinity [^{3}H]diazepam binding. *Proc. Natl. Acad. Sci. USA* **74**:3805–3809 (1977).
15. LeFur, G., N. Vaucher, M. L. Perrier, A. Flamier, J. Benavides, C. Renault, M. C. Dubroeucq, C. Gueremy, and A. Uzan. Differentiation between two ligands for peripheral benzodiazepine binding sites, [^{3}H]Ro-5-4864 and [^{3}H]PK11195 by thermodynamic studies. *Life Sci.* **33**:449–457 (1983).
16. Julou, L., J. C. Bardone, J. C. Blanchard, C. Garret, and J. M. Stutzman. Pharmacological studies on zopiclone. *Pharmacology* **27**(Suppl. 2):46–58 (1983).
17. Nicholson, A. N., and B. M. Stone. Zopiclone: sleep and performance studies in healthy man. *Pharmacology* **27**(Suppl. 2):92–97 (1983).
18. Gotfryd, M. A. A double-blind comparative study of suriclone, lorazepam and placebo. *Clin. Neuropharmacol.* **7**(Suppl. 1):626–627 (1984).
19. Costa, T., D. Rodbard, and C. B. Pert. Is the benzodiazepine receptor coupled to a chloride ion channel? *Nature* **277**:315–317 (1979).
20. Ninan, P. T., T. M. Insel, J. M. Cook, P. Skolnick, and S. M. Paul. Benzodiazepine receptor-mediated experimental "anxiety" in primates. *Science* **218**:1332–1334 (1982).
21. Tallman, J. F., J. W. Thomas, and D. W. Gallagher. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* **274**:383–385 (1978).
22. Olson, R. W. GABA-benzodiazepine-barbiturate interactions. *J. Neurochem.* **37**:1–13 (1981).
23. Salama, A. I. Tracazolate: a novel non-benzodiazepine anxiolytic, in *Pharmacology of Benzodiazepines* (E. Usdin, P. Skolnick, J. F. Tallman, D. Greenblatt, and S. M. Paul, eds.). Macmillan Press, London, 417–430, (1982).
24. Karobath, M., and P. Supavilai. Distinction of benzodiazepine agonists from antagonists by photoaffinity labeling of benzodiazepine receptors *in vitro*. *Neurosci. Lett.* **31**:65–69 (1982).
25. Mohler, H. Benzodiazepine receptors: differential interaction of benzodiazepine agonists and antagonists after photoaffinity labeling with flunitrazepam. *Eur. J. Pharmacol.* **80**:435–436 (1982).
26. Doble, A. Comparative thermodynamics of benzodiazepine receptor-ligand interactions in rat neuronal membranes. *J. Neurochem.* **40**:1605–1612 (1983).
27. Mohler, H., and J. G. Richards. Agonist and antagonist benzodiazepine receptor interactions *in vitro*. *Nature* **294**:763–765 (1981).
28. Quast, U., H. Mahlmann, and K.-O. Vollmer. Temperature dependence of the benzodiazepine-receptor interaction. *Mol. Pharmacol.* **22**:20–25 (1982).
29. Speth, R. C., G. J. Wastek, and H. I. Yamamura. Benzodiazepine receptors: temperature dependence of [^{3}H]flunitrazepam binding. *Life Sci.* **24**:351–358 (1979).
30. Chiu, T. H., D. M. Dryden, and H. C. Rosenberg. Kinetics of [^{3}H]flunitrazepam binding to membrane-bound benzodiazepine receptors. *Mol. Pharmacol.* **21**:57–65 (1982).
31. Quast, U., and H. Mahlmann. Interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor: evidence for a ligand-induced conformational change. *Biochem. Pharmacol.* **31**:2761–2768 (1982).
32. Mohler, H., W. Sieghart, P. Polc, E. Bonetti, and W. Hunkeler. Differential interaction of agonists and antagonists with benzodiazepine receptors, in *Pharmacology of Benzodiazepines* (E. Usdin, P. Skolnick, J. F. Tallman, D. Greenblatt, and S. M. Paul, eds.). Macmillan Press, London, 63–70 (1982).

Send reprint requests to: Rosario R. Trifiletti, Department of Neuroscience, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.