PICROTOXININ AND DIAZEPAM BIND TO TWO DISTINCT PROTEINS: FURTHER EVIDENCE THAT PENTOBARBITAL MAY ACT AT THE PICROTOXININ SITE¹

WILLIAM C. DAVIS AND MAHARAJ K. TICKU²

Division of Molecular Pharmacology, Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Abstract

α -[³H]Dihydropicrotoxinin (DHP) and [³H]diazepam binding proteins were solubilized from rat brain membranes with 1% Lubrol-Px. Gel filtration of the Lubrol-solubilized fraction revealed that [³H]DHP and [³H]diazepam bind to two distinct peaks with apparent molecular weights of 185,000 and 61,000, respectively. The signal-to-noise ratio of [³H]DHP binding to 185,000-dalton fractions was improved significantly. [³H]DHP bound to the 185,000-dalton fraction with two binding constants. Muscimol and pentobarbital, while enhancing [³H]diazepam binding to membrane and crude Lubrol-solubilized fractions, failed to enhance [³H]diazepam binding to the 61,000-dalton fraction. Pentobarbital inhibited the binding of [³H]DHP to the 185,000-dalton fraction with an IC₅₀ value of $60 \pm 12 \mu\text{M}$. The binding of [³H]DHP also was inhibited by several depressant and convulsant drugs which affect γ -aminobutyric acid (GABA)-mediated transmission. These results provide strong evidence that picrotoxinin and diazepam bind to two distinct proteins and that pentobarbital may act at the picrotoxinin-sensitive site of the benzodiazepine-GABA receptor-ionophore complex.

The γ -aminobutyric acid (GABA) receptor system appears to be composed of multiple components, including GABA recognition sites (Enna and Snyder, 1975; Olsen et al., 1979), benzodiazepine sites (Squires and Braestrup, 1977; Möhler and Okada, 1977), and picrotoxinin (α -dihydropicrotoxinin) sites (Ticku et al., 1978; Olsen et al., 1979). A variety of centrally acting drugs have been reported to affect GABAergic transmission (Nicoll, 1975; Barker and Ransom, 1978; Bowery and Dray, 1976; Scholfield, 1980; Tallman et al., 1980). To understand better the interaction of these drugs at the molecular level requires solubilization and purification of components of the benzodiazepine-GABA receptor-ionophore complex and subsequent reconstitution. Recently, GABA (Greenlee and Olsen, 1978), benzodiazepine (Yousufi et al., 1979; Lang et al., 1979; Gavish et al., 1979; Gavish and Snyder, 1981), and picrotoxinin (Davis and Ticku, 1980, 1981a) binding sites have been solubilized.

We have reported previously that a variety of depressant and convulsant drugs which affect GABAergic transmission inhibit the binding of a biologically active analogue of picrotoxinin, α -[³H]dihydropicrotoxinin (DHP) to rat brain membranes (Ticku et al., 1978; Ticku and

Olsen, 1978, 1979; Olsen et al., 1979). However, a major problem in [³H]DHP binding to membranes has been an unfavorable signal-to-noise ratio (Ticku et al., 1978; Ticku and Olsen, 1978). This ratio usually represents 1 part bound to 9 to 10 parts background. Recently, we improved this signal-to-noise ratio significantly by solubilizing the DHP binding sites with Lubrol-Px (Davis and Ticku, 1980, 1981a). In this paper, we report that we have purified the DHP binding receptors with a much improved signal-to-noise ratio. We also provide evidence that [³H]DHP and [³H]diazepam bind to two distinct proteins. Furthermore, we have investigated the interaction of the GABA agonists, muscimol and pentobarbital, with [³H]DHP and [³H]diazepam binding sites at subcellular and molecular levels.

Materials and Methods

[³H]Diazepam (76.8 Ci/mmol), [³H]DHP (29.96 Ci/mmol), and [³H]muscimol (10.3 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Nonradioactive benzodiazepines were gifts from Dr. W. Scott (Hoffmann-La Roche, Nutley, NJ). Stereoisomers of *N*-methyl-5-phenyl-5-propyl barbituric acid (MPPB) were gifts from Dr. Knabe (Saarland, West Germany) and 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBB) was a gift from Eli Lilly and Co. (Indianapolis, IN). Other chemicals were purchased from Sigma Chemical Co. (St.

¹ This work was supported by funds from National Institutes of Health Grant NS 15339. We thank Mrs. M. Wilson for excellent secretarial help.

² To whom correspondence should be addressed.

Louis, MO). Gel filtration supplies were purchased from Pharmacia (Piscataway, NJ).

Solubilization procedure. Male Sprague-Dawley rats (200 to 225 gm) were decapitated and their brains were removed. The mitochondrial plus microsomal ($P_2 + P_3$) fraction was prepared as described (Ticku et al., 1978). The $P_2 + P_3$ pellet was resuspended in buffer (0.2 M NaCl, 10 mM sodium phosphate, pH 7.0, at 4°C) and solubilized with 1% Lubrol-Px for 30 min as described (Davis and Ticku, 1981a). The suspension was centrifuged at $100,000 \times g$ for 45 min. The clear supernatant was removed, placed in dialysis tubing (exclusion limit, 12,000) and dialyzed against 250 ml of buffer for 4 hr at 0 to 4°C. This fraction is referred to as the crude Lubrol fraction. This fraction was used for binding studies as such or subjected to gel filtration as described below.

Gel filtration. Sephadex G-200 (fine) was allowed to swell in buffer containing 0.2 M NaCl, 10 mM sodium phosphate, pH 7.0, at 4°C. The hydrated gel and buffer were deaerated routinely under vacuum prior to use. The gel was poured into jacketed columns (1.5×84 or 2.5×40 cm). Crude Lubrol fraction (3 to 4 ml) was layered on the equilibrated column and eluted with the above buffer. Elute fractions (1 ml) were collected and ultraviolet absorption of the column elute was monitored at 280 nm with a recording spectrophotometer. Constant flow rates (25 ml/hr) were maintained by a pump system. The elution volumes (V_E) were corrected for the volume of tubing from the column tip to the collecting tube. Blue dextran (2000, Pharmacia), which was excluded completely from the gel interior, was used to determine the void volume (V_0). Tritiated water, which occupies both interior and exterior phases, was used to determine the total volume (V_T). For Stoke's radius and molecular weight determinations, Sephadex G-200 was calibrated with standard bovine serum albumin, aldolase, catalase, and ferritin. Column calibration was carried out routinely and the elution volumes of any standard protein usually agreed within ± 2.0 ml.

Binding studies. The binding of [3 H]DHP (Davis and Ticku, 1980, 1981a) and [3 H]diazepam (Davis and Ticku, 1981b) to the soluble receptors was measured by a centrifugation assay as described. This is a modification of the methods of Cuatrecasas (1972) and Leberman (1966). Briefly, aliquots (in triplicate) of the crude Lubrol fractions obtained by gel filtration were incubated with either 34 nM [3 H]DHP (15 min) or 1 nM [3 H]diazepam (30 min) with or without other drugs in a total incubation volume of 1 ml. Following incubation, 0.5 ml of 1% (w/v) bovine γ -globulin and 0.5 ml of 24% polyethylene glycol (PEG, 6000) were added to the vials. The vials were centrifuged at $48,000 \times g$ for 10 min. The pellet was washed rapidly with 5 ml of cold buffer, solubilized overnight with 0.3 ml of Soluene (Packard), and counted in 3 ml of scintillation fluid containing 5 gm of 2,5-diphenyloxazole per liter of toluene. Nonspecific binding was measured under identical conditions and in the presence of 100 μ M DHP for [3 H]DHP binding and 10 μ M diazepam for [3 H]diazepam binding. The binding of [3 H]DHP (Ticku et al., 1978) and [3 H]diazepam (Ticku, 1981) to membranes was measured as previously described. [3 H]Muscimol binding was studied by a similar centrifugation assay, using 10 nM [3 H]-

muscimol. Nonspecific binding for [3 H]muscimol was measured in the presence of 100 μ M GABA. The counting efficiency was $42 \pm 2\%$. Protein was measured by the method of Lowry et al. (1951).

Results

Table I shows that specific binding of [3 H]diazepam, [3 H]muscimol, and [3 H]DHP was present in the crude Lubrol fraction. We have reported recently that this fraction binds [3 H]DHP (Davis and Ticku, 1980, 1981a) and [3 H]diazepam (Davis and Ticku, 1981b) with binding constants and ligand specificity similar to that reported for membrane receptors.

Gel filtration of crude Lubrol fraction. When crude Lubrol-solubilized extract was chromatographed on Sephadex G-200, a major protein peak representing 60 to 70% of the applied protein appeared in the void volume (data not shown). After the void volume, 25 to 27% of the applied protein was eluted as two major protein peaks. Initial binding studies indicated that [3 H]DHP binding was associated with the high molecular weight peak (peak I) and that [3 H]diazepam binding was associated with the low molecular weight peak (peak II). The two peaks (without the void volume) were pooled and rechromatographed separately on Sephadex G-200. Following the second gel filtration, the two peaks were clearly separated (Fig. 1). In each case, the recovery of protein averaged greater than 90%. The elution profile shown in Figure 1 is the one obtained after the second gel filtration. The specific binding of [3 H]DHP was associated with peak I, and [3 H]diazepam binding was associated with peak II.

Table II shows the distribution of [3 H]DHP and [3 H]diazepam binding in the membranes, in the crude Lubrol fraction, and in peaks I and II. Specific [3 H]DHP and [3 H]diazepam binding was present in both the membranes and the crude Lubrol fraction. The specific binding of [3 H]DHP was improved significantly from $\sim 17\%$ in the membranes to $\sim 43\%$ in the crude Lubrol fraction (Table II, *a* and *b*).

TABLE I

Benzodiazepine-GABA receptor-ionophore complex ligand binding in the crude Lubrol-solubilized fraction

The binding of [3 H]diazepam, [3 H]muscimol, and [3 H]DHP to a soluble fraction was measured by PEG centrifugation assay. The specific binding of [3 H]muscimol represented $40 \pm 4\%$, and for [3 H]DHP, it was $38 \pm 4\%$ of the total pelleted radioactivity. The values represent the mean $\pm$ SD of a typical experiment done in triplicate. Similar results were replicated three times.

	In Absence of Excess Nonra- dioactive Ligand	In Presence of Excess Nonra- dioactive Ligand (i.e., Back- ground)	Displace- able Binding	Specific Binding
		<i>cpm</i>		<i>pmol/mg protein</i>
(a) [3 H]Diazepam, 1 nM	9,174 $\pm$ 169	4,609 $\pm$ 166	4,565	0.137
(b) [3 H]Muscimol, 10 nM	9,945 $\pm$ 100	5,694 $\pm$ 382	4,251	1.054
(c) α -[3 H]Dihydropicrotoxin, 34 nM	10,507 $\pm$ 138	4,982 $\pm$ 238	5,527	0.374

Following gel filtration, [^3H]DHP and [^3H]diazepam binding components could be separated (Table II, *c* and *d*). Thus, specific [^3H]DHP binding was present in peak I and [^3H]diazepam binding was present in peak II. Table IIb also shows that peak II, which is the [^3H]diazepam binding fraction, binds [^3H]DHP significantly in a non-specific (i.e., nondisplaceable) manner. In contrast, peak I, which binds [^3H]DHP specifically, had minimal non-specific binding. The signal-to-noise ratio of [^3H]DHP in the peak I fraction varied from 3.5 to 6 parts bound to 1 part background. Furthermore, [^3H]muscimol binding, which was present in the crude Lubrol-solubilized fraction (Table Ib), co-migrated with peak I (data not shown).

Molecular properties of peak I and peak II proteins. The molecular weights of peak I and peak II proteins were obtained by gel filtration (Fig. 2). Table III lists the molecular properties of peak I and II proteins. The

molecular weights of peaks I and II, as obtained by gel filtration, were $185,000 \pm 6,000$ and $61,000 \pm 5,000$ ($n = 3$), respectively. Comparison of these peaks with reference proteins (Fig. 2) indicates a Stoke's radius of 60.0 ± 1.7 Å for peak I and 20.8 ± 3.0 Å for peak II ($n = 3$). The Stoke's radius values for the two proteins were highly reproducible. The sedimentation coefficients ($s_{20,w}$) of these two peaks indicated values of 8.32 ± 3.0 for peak I and 8.23 ± 1.0 for peak II ($n = 3$).

Binding constants of [^3H]DHP binding to 185,000-dalton fraction. A typical Scatchard plot of [^3H]DHP binding to the 185,000-dalton fraction is shown in Figure 3. The Scatchard plots were curvilinear, suggesting heterogeneity of the DHP binding sites. However, curved Scatchard plots also could imply negative cooperativity or a two-step binding reaction. The binding constants of [^3H]DHP binding were obtained by subjecting the Scatchard data to one-ligand two non-interacting binding

Figure 1. Gel filtration elution profile on Sephadex G-200 of the 1% crude Lubrol-solubilized fraction from rat brain membranes. The data were obtained following the removal of the void volume, and the pooled fractions of *peaks I* and *II* were rechromatographed separately. *Peaks I* and *II* show the distribution of [^3H]DHP and [^3H]diazepam, respectively.

TABLE II

[^3H]Diazepam and [^3H]DHP binding in rat brain membranes, crude Lubrol extract, and purified fraction

[^3H]Diazepam (Ticku, 1981) and [^3H]DHP (Ticku et al., 1978) binding to membranes was measured by a filtration and centrifugation assay, respectively, as described. The binding of these ligands to the crude Lubrol and peak I and II fractions was measured by a centrifugation assay as described under "Materials and Methods." One nanomolar [^3H]diazepam and 34 nM [^3H]DHP were used. The results are the mean $\pm$ SD of one experiment (in triplicate). Similar results were replicated three times.

	[^3H]Diazepam Binding				[^3H]DHP Binding			
	Total Binding	Nonspecific Binding	Protein	Specific Binding	Total Binding	Nonspecific Binding	Protein	Specific Binding
	cpm		mg/assay	cpm/mg	cpm		mg/assay	cpm/mg
(a) Membranes	$1,554 \pm 35$	231 ± 65	0.073	18,123	$33,765 \pm 915$	$28,921 \pm 898$	1.900	$3,075 (0.17)^a$
(b) Crude Lubrol fraction	$9,557 \pm 161$	$3,686 \pm 147$	0.400	14,678	$14,316 \pm 360$	$10,009 \pm 118$	0.602	$7,178 (0.43)^a$
(c) Peak I	356 ± 10	323 ± 34	0.320	103	$6,429 \pm 95$	$1,404 \pm 65$	0.370	$13,581 (3.60)^a$
(d) Peak II	$5,992 \pm 166$	355 ± 15	0.310	14,990	$6,865 \pm 149$	$6,629 \pm 121$	0.301	684

^a Values in the parentheses are the signal-to-noise ratios.

Figure 2. Molecular weight determination of DHP and diazepam receptors by gel filtration. Molecular weights were determined by comparison with standard protein and were calculated by regression analysis.

TABLE III

Molecular properties of picrotoxinin and benzodiazepine receptors

These parameters were obtained by Sephadex G-200 gel filtration. The standards run in parallel are shown in Figure 2. The values are the mean $\pm$ SD of three separate experiments.

	Stoke's Radius	Sedimentation Coefficient	Molecular Weight ^a
	$\text{\AA}$	$S_{20,w}$	
Peak I (DHP receptor)	63.00 ± 1.7	8.32 ± 3.0	$185,000 \pm 6,000$
Peak II (Benzodiazepine receptor)	20.80 ± 3.0	8.23 ± 1.0	$61,000 \pm 5,000$

^a Molecular weights were determined by gel filtration.

sites, according to the curve-fitting procedure of Feldman (1972). The high affinity site had a K_{D1} value of $0.054 \pm 0.009 \mu\text{M}$ and a $B_{\text{max}1}$ of $0.930 \pm 1.55 \text{ pmol/mg}$ of protein, and the low affinity site had a K_{D2} value of $0.546 \pm 0.065 \mu\text{M}$ and a $B_{\text{max}2}$ of $7.178 \pm 0.168 \text{ pmol/mg}$ of protein (values are mean $\pm$ SD of four separate experiments, each done in triplicate).

Binding of benzodiazepines to 61,000-dalton fraction. [^3H]Diazepam binding to the 61,000-dalton fraction was saturable. [^3H]Diazepam and [^3H]flunitrazepam bound to this fraction with apparent K_D values of 6.11 ± 0.98 and $1.55 \pm 0.03 \text{ nM}$ ($n = 2$). [^3H]Diazepam binding was inhibited by clonazepam with an IC_{50} value of 1 nM and by diazepam with an IC_{50} value of 8 nM .

Effect of muscimol and pentobarbital on [^3H]diazepam binding. Table IV shows that muscimol and pentobarbital enhance [^3H]diazepam binding to membrane and crude Lubrol fractions. However, these ligands did not enhance [^3H]diazepam binding to the 61,000-dalton fraction (peak II). Table V shows that specific [^3H]muscimol binding was present in the crude Lubrol and

185,000-dalton fractions but not in the 61,000-dalton fraction. These results indicate that muscimol binding sites had migrated with the 185,000-dalton fraction (peak I). [^3H]Muscimol binding to the 185,000-dalton fraction was displaced by GABA ($\text{IC}_{50} = 0.1 \mu\text{M}$) and (+)-bicuculline ($\text{IC}_{50} = 4 \mu\text{M}$) but not by β -alanine and nipecotic acid.

Effect of depressant and convulsant drugs on [^3H]DHP binding to 185,000-dalton fraction. Table VI compares the IC_{50} values of some depressant and convulsant drugs on the binding of [^3H]DHP to the 185,000-dalton and membrane fractions. [^3H]DHP binding was inhibited by convulsants, such as picrotoxinin, DHP, RO5-3663, isopropyl bicyclopophosphate, cyclohexylidene barbituric acid (CHEB), and pentylenetetrazole. Depressant barbiturates, such as hexobarbital, pentobarbital, and secobarbital, also inhibited [^3H]DHP binding. Figure 4 shows a dose-dependent displacement of [^3H]DHP binding by pentobarbital. The IC_{50} value for pentobarbital inhibition of [^3H]DHP binding was $60 \pm 12 \mu\text{M}$ (mean $\pm$ SD, $n = 3$). [^3H]DHP binding to the 185,000-dalton fraction was not inhibited by GABA agonists and other unrelated drugs (Table VI).

Discussion

We have solubilized the picrotoxinin binding receptor from rat brain membranes successfully. Our results clearly indicate that this protein is very closely associated with the benzodiazepine receptor in the membranes and in the crude Lubrol-solubilized fraction (under the conditions described under "Materials and Methods"). However, following gel filtration, this protein could be dissociated from the benzodiazepine-GABA receptor-ionophore complex. Additionally, we have resolved a major problem of high nonspecific [^3H]DHP binding usually

Figure 3. Scatchard plot of [^3H]DHP binding to 185,000-dalton fractions. [^3H]DHP was varied for the low concentration points (4 to 100 nM) and for the high points (>100 nM); the concentration of nonradioactive DHP was varied, keeping [^3H]DHP constant at 100 nM. Background obtained in the presence of 10^{-4} M DHP was subtracted from each point. Binding data were plotted as a Scatchard plot. K_D and B_{max} values were obtained by Feldman (1972) analysis and are presented in the text.

TABLE IV
Effect of pentobarbital and muscimol on [3 H]diazepam binding

The binding of [3 H]diazepam to the crude Lubrol and 61,000-dalton fractions was measured by a centrifugation assay as described under "Materials and Methods." The results are the mean $\pm$ SD of one experiment (in triplicate) in which the 61,000-dalton fraction was obtained by gel filtration of the same crude Lubrol fraction used for the binding studies. Similar results were replicated four times. The values in parentheses are the enhancement over base line control values.

Treatment	Specific [3 H]Diazepam Binding		
	Membrane ^a	Crude Lubrol Fraction	61,000-Dalton Fraction
		fmol/mg protein	
Control (100%)	119.3 $\pm$ 8.7	115.7 $\pm$ 8.4	193.0 $\pm$ 5.4
+10 ⁻⁷ M muscimol	182.4 $\pm$ 8.2 ($\uparrow$ 53%)	N.T. ^b	182.7 $\pm$ 6.8
+10 ⁻⁵ M muscimol	330.9 $\pm$ 6.9 ($\uparrow$ 177%)	265.3 $\pm$ 11.4 ($\uparrow$ 119%)	195.6 $\pm$ 4.8
+10 ⁻⁴ M pentobarbital	152.5 $\pm$ 7.1 ($\uparrow$ 28%)	138.8 $\pm$ 4.6 ($\uparrow$ 20%)	187.3 $\pm$ 6.9
+10 ⁻³ M pentobarbital	228.9 $\pm$ 9.7 ($\uparrow$ 92%)	183.6 $\pm$ 6.8 ($\uparrow$ 59%)	193.1 $\pm$ 4.8

^a Values for membranes are from Ticku (1981).

^b N.T., not tested.

TABLE V
[3 H]Muscimol binding in crude Lubrol, peak I, and peak II fractions

Muscimol binding was measured by a centrifugation assay using 10 nM [3 H]muscimol. The values in parentheses are the protein concentrations per assay. The results are the mean $\pm$ SD of one experiment (in triplicate). Similar results were replicated twice. [3 H]Muscimol binding to the crude Lubrol fraction and peak I fraction was displaced in a dose-dependent manner by (+)-bicuculline.

	[3 H]Muscimol Binding		
	Total Binding	In Presence of 100 μ M GABA	Specific Binding
		cpm/assay	
Crude Lubrol fraction (0.48 mg)	5,108 $\pm$ 81	2,473 $\pm$ 23	2,635
Peak I (0.37 mg)	2,827 $\pm$ 114	128 $\pm$ 17	2,699
Peak II (0.30 mg)	1,647 $\pm$ 188	1,303 $\pm$ 110	344

TABLE VI
IC₅₀ values for inhibition of [3 H]DHP binding to membrane and 185,000-dalton fractions

IC₅₀ values were determined using five to seven concentrations of the ligand. Results are the mean values from two to three experiments for the 185,000-dalton fraction. Values for the membranes are from Olsen et al. (1979), Ticku et al. (1978), and Ticku and Olsen (1978).

Ligand	IC ₅₀	
	185,000-Dalton Fraction	Membrane Fraction
	μ M	
($\pm$)-DMBB	0.02	0.01
RO5-3663	0.20	0.40
CHEB	0.40	0.70
Picrotoxinin	0.45	0.40
DHP	1.10	1-2
($\pm$)-Hexobarbital	3.00	12
($\pm$)-Secobarbital	10.00	5
Isopropyl bicyclopophosphate	8.00	10
Pentylentetrazole	40.00	>10
($\pm$)-Pentobarbital	60.00	50
Phenobarbital	300.00	400
GABA	>200.00	>200
Muscimol	>200.00	>200
Haloperidol	>100.00	N.T. ^a
Chlorpromazine	>100.00	N.T.
Acetylcholine	>200.00	N.T.
L-Glutamate	>100.00	N.T.

^a N.T., not tested.

observed with membrane preparations (Ticku et al., 1978; Ticku and Olsen, 1978). As indicated in Table II, a major contribution to the high nonspecific [3 H]DHP binding appears to be due to its ability to stick nonspecifically to the benzodiazepine binding protein. Thus, most of the nonspecific [3 H]DHP binding observed in the crude Lubrol fraction (Table IIb) could be detected in peak II (i.e., the diazepam binding fraction). In contrast, peak I, which is the [3 H]DHP binding protein, had minimal nonspecific binding. Furthermore, unmasking of the high affinity DHP binding site also improved the signal-to-noise ratio significantly.

Our results also provide evidence for the first time that [3 H]DHP and [3 H]diazepam bind to two distinct proteins with apparent molecular weights of 185,000 and 61,000 daltons. However, these values may be overestimated due to the residual detergent associated with these proteins. Under our experimental conditions of protein concentration and ionic composition, the association-dissociation phenomenon and the possibility of the proteins reacting to the column support were minimized. Therefore, the molecular weight of the fractions determined by gel filtration are relatively accurate. Other investigators have reported molecular weights of ~200,000 daltons (Yousufi et al., 1979) and 50,000 to 60,000 (Möhler et al., 1980; Sieghart and Karobath, 1980) and 55,000 to 62,000 (Gavish and Snyder, 1981) daltons for the benzodiazepine receptor.

GABA agonists have been reported to enhance [3 H]-diazepam binding to membranes (Tallman et al., 1980) and to solubilized fractions (Gavish and Snyder, 1980). In the present study, we find that, while GABA agonists, such as muscimol, enhance [3 H]diazepam binding to membranes and the crude Lubrol-solubilized fraction, no enhancement was observed in peak II following gel filtration of the Lubrol-solubilized fraction. Our results also show that, while specific [3 H]muscimol binding was present in the crude Lubrol fraction, it was not associated with the peak II fraction (i.e., diazepam binding fraction). However, [3 H]muscimol binding activity co-migrated with peak I (i.e., the DHP binding fraction). Muscimol binding appeared to have the right properties, since it was displaced by GABA and (+)-bicuculline but not by β -alanine and nipecotic acid. Recently, several laboratories have attempted to establish if GABA and benzodiazepine binding proteins are the same or distinct (Gavish and Snyder, 1981; Massotti et al., 1981).

Figure 4. Displacement of specific [³H]DHP binding to the 185,000-dalton fraction by pentobarbital. The values are the mean $\pm$ SD of two experiments.

DHP binding to the peak I protein exhibited two binding sites, in contrast to only one site observed for the membrane receptors (Ticku et al., 1978; Olsen et al., 1979). However, we recently reported that [³H]DHP bound to the crude Lubrol-solubilized fraction with two affinity constants (Davis and Ticku, 1980, 1981a). The 185,000-dalton fraction had a K_{D1} value of 0.054 ± 0.009 μ M and the low affinity site had a K_{D2} of 0.546 ± 0.065 μ M. The low affinity site had a K_D much tighter than the Lubrol-solubilized fraction ($K_{D2} \sim 1.85$ μ M; Davis and Ticku, 1980, 1981a) or the membrane receptors ($K_D \sim 1$ to 2 μ M; Ticku et al., 1978). The reason for the heterogeneity of the DHP binding sites is not clear. We have reported earlier that some ligands displace DHP binding from membranes with shallow displacement curves (Ticku and Olsen, 1978), suggesting heterogeneity of the DHP binding sites. The possibility that uncoupling of the effector complexes may have occurred cannot be ruled out.

We have reported previously that the picrotoxinin site at the benzodiazepine-GABA receptor-ionophore complex is a site of action for some depressant and convulsant drugs that affect GABAergic transmission (Ticku and Olsen, 1978, 1979, 1980; Olsen et al., 1979; Ticku, 1980). In the present study, we find that depressant and convulsant drugs inhibit the binding of [³H]DHP to the 185,000-dalton fraction (Table VI). The ligand specificity of [³H]DHP binding to 185,000-dalton fraction is not altered significantly relative to membrane receptors.

Like GABA agonists, depressant barbiturates, such as pentobarbital, enhance [³H]diazepam binding by increasing its affinity for receptors to membranes (Leeb-Lundberg et al., 1980; Ticku, 1981) and to crude Lubrol fraction (Davis and Ticku, 1981b; this study). This enhancement was blocked by picrotoxinin. The inability of pentobarbital to enhance [³H]diazepam binding to the 61,000-dalton fraction and its ability to inhibit [³H]DHP binding to the 185,000-dalton fraction strongly suggests that pentobarbital acts at the picrotoxinin site. The IC_{50} value for pentobarbital inhibition of DHP binding was similar to that reported for membranes (Ticku and Olsen, 1978).

These results are consistent with our hypothesis that the picrotoxinin site may be a receptor for barbiturates (Ticku, 1980). The ability of barbiturates with opposite pharmacological activities (i.e., depressant and convulsant) to inhibit DHP binding to membranes, the crude Lubrol fraction, and the 185,000-dalton fraction suggests that the picrotoxinin site may be an allosteric site at the benzodiazepine-GABA receptor-ionophore complex. However, further experimental evidence will be needed to clarify this and to establish the physiological significance of this site to GABAergic transmission.

References

- Barker, J. L., and B. R. Ransom (1978) Pentobarbital pharmacology of mammalian central neurons grown in tissue culture. *J. Physiol. (Lond.)* 280: 355-372.
- Bowery, N. G., and A. Dray (1976) Barbiturate reversal of amino acid antagonisms produced by convulsant agents. *Nature* 264: 276-278.
- Cuatrecasas, P. (1972) Isolation of the insulin receptor of liver and fat-cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* 69: 318-322.
- Davis, W. C., and M. K. Ticku (1980) Solubilization of the dihydropicrotoxinin binding sites. *Eur. J. Pharmacol.* 68: 397-399.
- Davis, W. C., and M. K. Ticku (1981a) Solubilization of the picrotoxinin binding receptor from mammalian brain. *J. Neurochem.* 36: 1572-1579.
- Davis, W. C., and M. K. Ticku (1981b) Pentobarbital enhances [³H]-diazepam binding to soluble receptors at the benzodiazepine-GABA receptor-ionophore complex. *Neurosci. Lett.* 23: 209-213.
- Enna, S. H., and S. H. Snyder (1975) Properties of γ -aminobutyric acid binding to receptor sites in rat central nervous system. *Brain Res.* 100: 81-97.
- Feldman, H. A. (1972) Mathematical theory of complex ligand-binding system at equilibrium: Some methods of parameter fitting. *Anal. Biochem.* 48: 317-338.
- Gavish, M., and S. H. Snyder (1980) Soluble benzodiazepine receptors: GABAergic regulation. *Life Sci.* 26: 579-582.
- Gavish, M., and S. H. Snyder (1981) γ -Aminobutyric acid and benzodiazepine receptors: Copurification and characterization. *Proc. Natl. Acad. Sci. U. S. A.* 78: 1939-1942.
- Gavish, M., R. S. L. Chang, and S. H. Snyder (1979) Solubili-

- zation of histamine H-1, GABA and benzodiazepine receptors. *Life Sci.* 25: 783-790.
- Greenlee, D., and R. W. Olsen (1978) Solubilization of γ -aminobutyric acid receptor from mammalian brain. *Biochem. Biophys. Res. Commun.* 88: 380-387.
- Lang, B., E. -A. Barnard, L. Chang, and O. J. Dolly (1979) Putative benzodiazepine receptors: A protein solubilization from brain. *FEBS Lett.* 104: 149-153.
- Leberman, R. (1966) The isolation of plant virus by means of simple coacervates. *Virology* 30: 341-347.
- Leeb-Lundberg, F., A. Snowman, and R. W. Olsen (1980) Barbiturate receptor sites are coupled to benzodiazepine receptors. *Proc. Natl. Acad. Sci. U. S. A.* 77: 7468-7472.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Massotti, M., A. Guidotti, and E. Costa (1981) Characterization of benzodiazepine and γ -aminobutyric recognition sites and their endogenous modulators. *J. Neurosci.* 1: 409-418.
- Möhler, H., and T. Okada (1977) Benzodiazepine receptor: Demonstration in the central nervous system. *Science* 198: 849-851.
- Möhler, H., M. K. Battersby, and J. G. Richards (1980) Benzodiazepine receptor protein identified and visualized in brain tissue by photoaffinity label. *Proc. Natl. Acad. Sci. U. S. A.* 77: 1666-1670.
- Nicoll, R. A. (1975) Pentobarbital action on frog motoneurons. *Brain Res.* 96: 119-123.
- Olsen, R. W., M. K. Ticku, D. Greenlee, and P. VanNess (1979) GABA receptor and ionophore binding sites: Interaction with various drugs. In *GABA—Neurotransmitters*, P. Krogsgaard-Larsen, J. Scheel-Kruger, and H. Kofod, eds., pp. 165-178, Munksgaard, Copenhagen.
- Scholfield, C. N. (1980) Convulsants antagonize inhibition in the olfactory cortex slices. *Naunyn Schmiedbergs Arch. Pharmacol.* 314: 29-36.
- Sieghart, W., and M. Karobath (1980) Molecular heterogeneity of benzodiazepine receptors. *Nature* 286: 285-287.
- Squires, R. F., and C. Braestrup (1977) Benzodiazepine receptors in rat brain. *Nature* 266: 732-734.
- Tallman, J., S. M. Paul, P. Skolnick, and D. W. Gallager (1980) Receptors for the age of anxiety: Pharmacology of the benzodiazepines. *Science* 207: 274-281.
- Ticku, M. K. (1980) Is the picrotoxinin binding site at the GABA synapse a site of action of barbiturates. *Brain Res. Bull. (Suppl. 2)* 5: 919-923.
- Ticku, M. K. (1981) Interaction of depressant, convulsant and anticonvulsant barbiturates with [3 H]-diazepam binding site at the benzodiazepine-GABA receptor-ionophore complex. *Biochem. Pharmacol.* 30: 1573-1579.
- Ticku, M. K., and R. W. Olsen (1978) Interaction of barbiturates with dihydropicrotoxinin binding sites related to the GABA receptor-ionophore complex. *Life Sci.* 22: 1643-1652.
- Ticku, M. K., and R. W. Olsen (1979) Cage convulsants inhibit picrotoxinin binding. *Neuropharmacology* 18: 315-318.
- Ticku, M. K., and R. W. Olsen (1980) Picrotoxinin binding sites at the GABA synapse: A target for direct action. *Brain Res. Bull. (Suppl. 2)* 5: 213-216.
- Ticku, M. K., M. Ban, and R. W. Olsen (1978) Binding of [3 H]- α -dihydropicrotoxinin, a γ -aminobutyric acid synaptic antagonist, to rat brain membranes. *Mol. Pharmacol.* 14: 391-402.
- Yousufi, M. A. K., J. W. Thomes, and J. F. Tallman (1979) Solubilization of benzodiazepine binding site from rat cortex. *Life Sci.* 25: 463-470.