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Biochemical Characterization of the ^{3}H-Diazepam Binding Site in an American Cockroach, Periplaneta americana, Head Membrane Preparation

presented by

Kevin Leigh Blair

has been accepted towards fulfillment
of the requirements for

M.S. degree in Entomology

Major professor

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Date

BIOCHEMICAL CHARACTERIZATION OF THE ^{3}H-DIAZEPAM BINDING SITE IN AN AMERICAN COCKROACH, PERIPLANETA AMERICANA, HEAD MEMBRANE PREPARATION

By

Kevin Leigh Blair

A THESIS

Submitted to
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MASTER OF SCIENCE

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1985

ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF THE ^{3}H-DIAZEPAM BINDING SITE IN AN AMERICAN COCKROACH, PERIPLANETA AMERICANA, HEAD MEMBRANE PREPARATION

by

Kevin Leigh Blair

A ^{3}H-diazepam binding assay was used to characterize the benzodiazepine binding site in membranes isolated from the head of the American cockroach, Periplaneta americana. The binding sites are one of the "peripheral type" since RO5-4864 was the most potent benzodiazepine tested and clonazepam and its 3-methyl analog, RO11-3128, were the least potent. In agreement with this conclusion, GABA did not stimulate ^{3}H-diazepam binding. Titration curves demonstrated that the specific binding was predominately to low affinity (uM range) sites. Most all of the drugs tested, that exhibited significant competitive activity are known to block voltage sensitive sodium channels through the batrachotoxin sensitive site. Chlordimeform was the most potent pesticide tested. DDT and pyrethroids had no statistically significant effect on binding, indicating that they interact at a site different from diazepam and chlordimeform. Calcium chelation by EGTA irreversibly altered the conformation of the binding site. Thus diazepam and chlordimeform may be exerting their actions (at least in part) through voltage sensitive sodium channels.

Zu BOBO, dem Ersten

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the Lord God for a challenging project and a curiosity to match.

Also, for providing me with an excellent teacher, Dr. Fumio Matsumura. Your suggestions and advice were most beneficial.

I also wish to thank Dr. Jim Miller, Dr. Ralph Pax, and Dr. Matt Zabik for the ideas, encouragement and knowledge that you have conveyed to me.

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INTRODUCTION

The benzodiazepines are a class of neuroactive drugs developed for their anxiolytic, anticonvulsant, sedative, and muscle relaxant properties in mammals (1, 2). Stereospecific, high affinity (3, 4) and low affinity (5) receptors have been identified in mammalian brain and are termed as "CNS type". Another high affinity receptor, pharmacologically distinct from the above type, has also been identified. It has been demonstrated in several mammalian peripheral tissues: e.g., lung, liver, and kidney (6), as well as in the brain (7), and is termed as "peripheral type". The "CNS type" and "peripheral type" high affinity receptors are differentiated by their different affinities for the benzodiazepines clonazepam and RO5-4864. The high affinity "peripheral type" receptor has low affinity for clonazepam and high affinity for RO5-4864 (see 8 for review). The low affinity "CNS type" receptor has only a marginally higher affinity for clonazepam (5). Only the high affinity "CNS type" receptor correlates well with the efficacy of benzodiazepines to potentiate the action of gamma-amniobutyric acid (GABA), their major molecular and pharmacological action (see 9, 10 for reviews). Indeed, the GABA receptor and high affinity "CNS type" benzodiazepine receptor are on the same protein (11).

GABA is the major inhibitory neurotransmitter in the mammalian brain. GABA mediated synapses may comprise as much as 30-50 percent of

the total synapses in the brain (12). GABA is also an inhibitory transmitter at the arthropod neuromuscular junction (13, 14). GABAergic function has also been demonstrated in the central nerve cord of the American cockroach (15, 16). GABA's inhibitory action is predominately through the activation of chloride channels. This GABA induced conductance change can be antagonized competitively by bicuculline and noncompetitively by picrotoxinin (17).

While the importance of GABAergic systems in mammals and the arthropod neuromuscular junction has long been recognized, the role of GABA in insect CNS functioning has not received extensive investigation. It has been reported that the picrotoxinin receptor is the major site of action for the cyclodiene type pesticides and gama-hexachlorocyclohexane (γ-HCH) in insects (see 18 for review). These pesticides also interact with the picrotoxinin receptor in the mammalian brain (19, 20). Avermectin Bla, a microbially derived pesticide, has been found to potentiate GABAergic actions in preparations of lobster neuromuscular junction (21), nematode nerves (22), and rat brains (23, 24). These interspecies similarities indicate evolutionary conservation of these components in the GABA system.

Type II pyrethoids act as partial antagonists to the ^{35}S-t-butylbicyclophosphorothionate (^{35}S-TBPS) binding site in the mammalian brain (25). TBPS is a picrotoxinin receptor agonist (26). ^{3}H-RO5-4864 is also displaced by type II pyrethroids in the mammalian

brain (27). Diazepam delayed the onset of type II pyrethroid toxicity in mouse and American cockroach, but not that of type I pyrethroids (28). Benzodiazepine receptors were thought to be nonexistent in invertebrates (29), but have recently been demonstrated in the housefly thorax (30) and housefly head (31). Binding to the thoracic receptor was enhanced by GABA, but the ligand specificity was quite different from that of the mammalian GABA/benzodiazepine complex. Type II pyrethroids, at high uM concentrations, enhanced ^{3}H-flunitrazepam binding in the housefly head. Chlordimeform, an acaricide, displaced ^{3}H-diazepam in an American cockroach head preparation at 100 uM. Diazepam was also observed to cause a transient excitation, then a block of movement in the German cockroach (32).

Thus there is impetus to better understand the benzodiazepine receptor(s) in insects and its (their) potential interactions with a GABA related system. The purpose of this study is to examine the basic biochemical characteristics of the benzodiazepine receptor(s) in the head of the American cockroach. Special attention has been paid to the specificity of interactions with a wide range of neuroactive agents.

MATERIALS AND METHODS

Materials

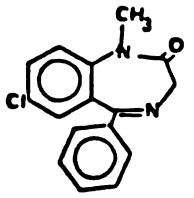
Animals and Chemicals

Animals

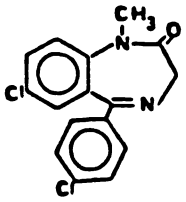
The American cockroaches (Periplaneta americana L.) were taken from cultures maintained by this laboratory for several years. New Zealand albino rabbits were obtained from a licensed dealer in Grand Rapids, Michigan.

Chemicals

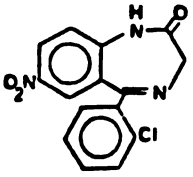
Chemicals were obtained from the following sources: RO5-4864, Dr. J. Bennett (Pharmacology, Michigan State University); praziquantel and RO11-3128, Dr. R. Pax (Zoology, Michigan State University); all other benzodiazepines, Dr. H. Moehler (Hoffman La Roche and Co.); Guthion®, Dr. M. Zabik (Michigan State University); cis and trans-permethrin, Environmental Protection Agency; cypermethrin, FMC; decamethrin, Roussel UCLAF; γ-HCH, Hooker Chemical company; Baygon®, Chemagro Corporation. Chlordimeform was synthesized in this lab by the condensation of 4-chloro-o-toluidine and dimethyl formamide. ^{3}H-diazepam (78.9 Ci/mmole) was purchased from New England Nuclear. All other chemicals were purchased from commercial sources. The structures of the chemicals studied are shown in Figure 1.



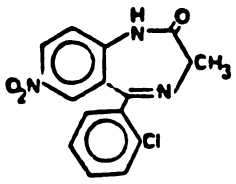
Diazepam



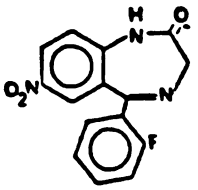
RO5-4864



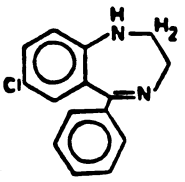
Clonazepam



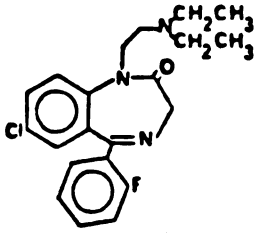
RO11-3128



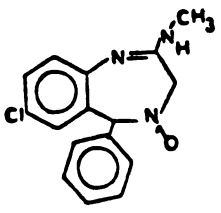
Flunitrazepam



Medazepam

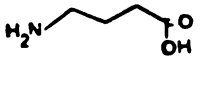


Flurazepam

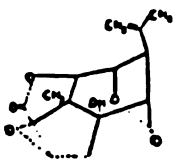


Chlordiazepoxide

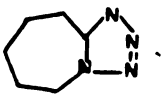
Figure 1. Chemical structures of the neuroactive agents studied.



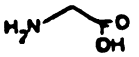
GABA



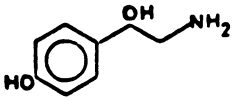
Picrotoxinin



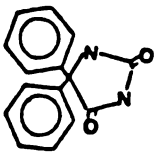
Pentylenetetrazol



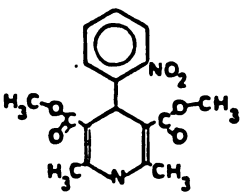
Glycine



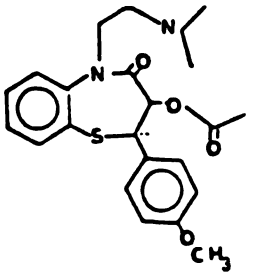
Octopamine



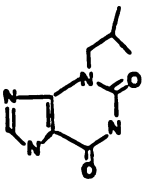
Phenytoin



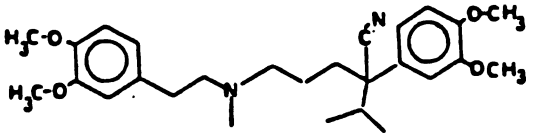
Nifedipine



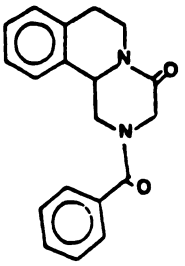
Diltiazem



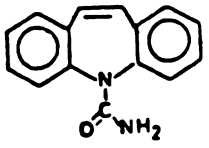
3-isobutyl-1-methyl xanthine



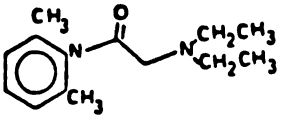
Verapamil



Praziquantel

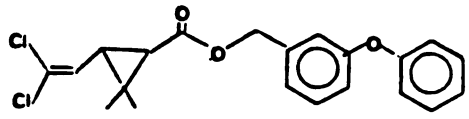


Carbamazepine

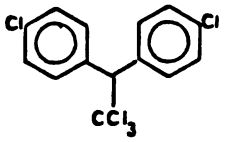


Lidocaine

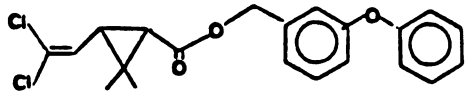
Figure 1 Cont.



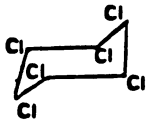
Trans-permethrin



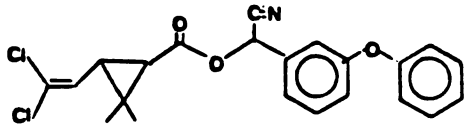
DDT



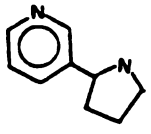
Cis-permethrin



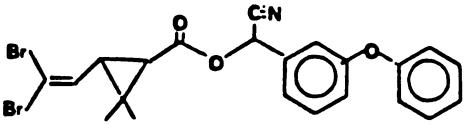
γ-HCH



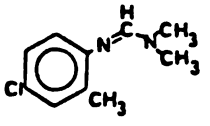
Cypermethrin



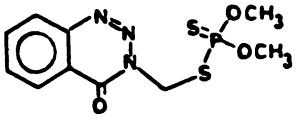
Nicotine



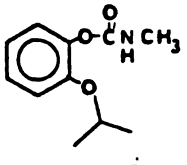
Decamethrin



Chlordimeform



Guthion R



Baygon R

Figure 1 Cont.

METHODS

Tissue Preparation

American Cockroach

The heads of adult male American cockroaches were homogenized (1 ml/head) in ice-cold modified Van Harrevelds saline (205 mM NaCl, 5.4 mMKCl, 13.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.7) (33) plus 0.3 mM phenylmethylsufonyl floride (except where noted) with a glass-glass homogenizer (Pyrex®). The homogenate was further homogenized with a Teflon®-glass homogenizer (Thomas type C) at 800-1000 RPM, 6-8 strokes. This homogenate was then centrifuged at 1000g for 10 minutes at 4 C. The supernatant was collected, filtered through glass wool and stored for later use. The pellet was resuspended in 0.6 ml/head of the same buffer and homogenized with glass-glass (Pyrex®) and then Teflon® glass (Wheaton 30 ml) homogenizers as before. This homogenate was centrifuged and collected as before. The supernatants were combined and centrifuged at 100,000g for 30 minutes. Thereafter, the supernatant was discarded and the inside of the centrifuge tube was wiped with tissue paper. The pellet was resuspended with a Teflon®-glass homogenizer (Wheaton 30 ml) as before in 5 mM Tris-HCl buffer (pH, 7.1) (except where noted) to give the equivalent of 1 head/500 ul. The preparation was made fresh on the day of the experiment.

Rabbit

Rabbits were killed by over-etherization and cardiac puncture. The brains and kidneys were removed, chopped, and stored in 0.32 M sucrose at -80^{0} On the day of the experiment, the samples of brains or kidneys were thawed, minced, and homogenized in 20 volumes of 0.32 M sucrose with a Teflon®-glass homogenizer (Wheaton 30 ml) as before. The homogenate was centrifuged at 1000g for 10 minutes. The supernatant was recentrifuged at 144,000g for 45 minutes. The supernatant was discarded and the pellet was suspended in 50 mM Tris-HCl (pH, 7.4), with a Teflon®-glass homogenizer (Wheaton 30 ml) as before, to give a protein concentration of approximately 4 mg/500 ul.

Binding Assay

Experiments were conducted in 5 ml glass culture tubes in triplicate, in an ice bath. The tissue homogenate was allowed to stabilize for 30-40 minutes. To 200 ul of 5mM Tris-HCl (pH 7.1) (or buffer plus treatment), 500 ul of the homogenate was added, and the system was allowed to stabilize for another 30-40 minutes. An unlabeled ligand of interest in 1 ul of dimethyl sulfoxide (DMSO for control) was added and the radiolingand binding was initiated by the addition of ^{3}H-diazepam in 300 ul of buffer to give a final concentration of 3.5-3.7 nM ^{3}H-diazepam. The assay was terminated after 40 minutes (except where noted) by dilution with 4 ml chilled buffer and rapid filtration through

Whatman GF-B filters. The filter was washed once with 5 ml of the chilled buffer. The filters were then dried, the protein solubilized in 350 ul 0.2 M NaOH over night, and the radioactivity determined by conventional liquid scintillation spectroscopy. Protein was determined by the Lowry method (34).

Analysis

Specific binding is defined as the difference between total binding and binding in the presence of 100 uM unlabeled diazepam. Percent displacement is the binding in the presence of the ligand of interest divided by the specific binding of the diazepam control. The data were analyzed by the Rank Sum Test or by Random Analysis of Variance. The arc sin $\sqrt{x}$ conversion was used to normalize the data. The Student Newman Keal multiple comparisons procedure was used to determine significance in the Random Analysis of Variance.

RESULTS

Transmission electron micrographs of the American cockroach head preparation showed it to be comprised predominantly of vesiculated membranes, 50-200 nm in diameter (micrograph not shown). Very few mitocondria were observed. Specific binding typically comprised 30-40 percent of the total binding. The contribution of CNS and muscular tissues to the specific binding was studied. The specific binding to the superoesophageal and suboesophageal ganglia was 8.2 $\pm$ 2.0 fmol ^{3}H-Diazepam/head. The muscular components contributed 26.0 $\pm$ 8.2 fmol ^{3}H-Diazepam/head. Thus on a per head basis, the musculature contributes the majority of the binding. However, on a mg protein basis, the CNS components bind more, 61.0 $\pm$ 14.9 fmol ^{3}H-Diazepam/mg protein and 33.4$\pm$ 10.5 fmol ^{3}H-Diazepam/mg protein for CNS and musculature respectively. The filter accounted for 50-60 percent of the nonspecific binding.

Several benzodiazepines were tested for their ability to displace ^{3}H-diazepam (Table 1). The most potent was RO5-4864 with a reduction of 107 $\pm$ 5.5%. Medazepam was quite potent with a reduction of 78 $\pm$ 14%. Chlordiazepoxide, flunitrazepam, and flurazepam were equally potent with a reduction of 45 $\pm$ 4.9, 44 $\pm$ 6.5, and 42 $\pm$ 6.7% respectively. Clonazepam was rather weak with only 17 $\pm$ 9.4%. The antischistosomal drug RO11-3128 actually increased ^{3}H-diazapam binding by 24 $\pm$ 7.6%.

TABLE 1.

Reduction of H-Diazepam Specific Binding to Membranes from the Head of American Cockroach by Various Benzodiazepines

Benzodiazepine	Concentration (uM)	Reduction, % of Control[a]	
Diazepam	100	100[b]	A
RO5-4864	100	107 ± 5.5	B
Medazepam	100	78 ± 14	C
Chlordiazepoxide	100	45 ± 4.9	D
Flunitrazepam	100	44 ± 6.5	D
Flurazepam	100	42 ± 6.7	D
Clonazepam	100	17 ± 9.4	E
RO11-3128	100	-24 ± 7.6	F

a Each mean is of three separate experiments performed in triplicate ± standard deviation. Values followed by the same letter are not significantly different by Student Newman Keal multiple comparison procedures at $p<.05$.

b Normalized control.

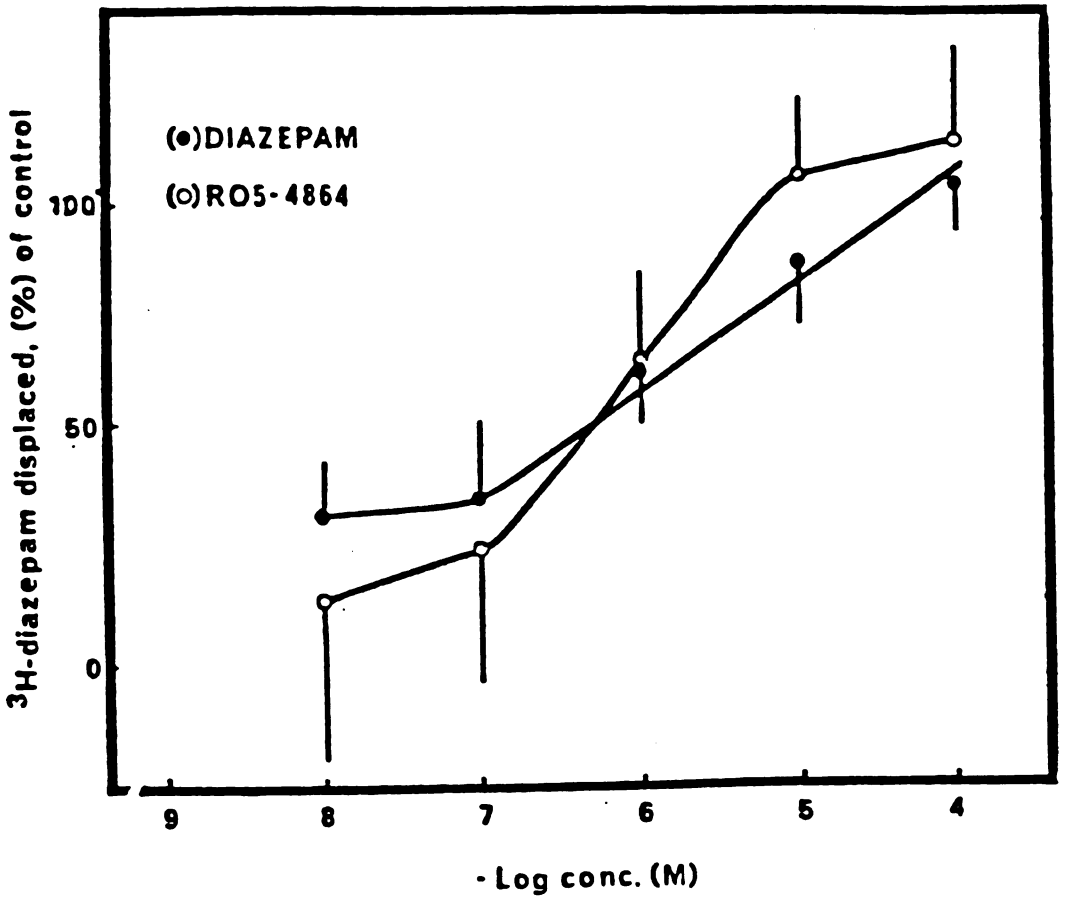


Figure 2. Titration curves of (•) diazepam and (o) RO5-4864. Each symbol and verticle bar is the mean and standard deviation of three replicates of one experiment. ^{3}H-diazepam concentration was 3.6 n<u>M</u>.

Titration curves of diazepam and RO5-4864 were then obtained (Figure 2). The curve for diazepam indicates the presence of a small saturable component below 100 nM. Above 100 nM, the curve indicates no saturation up to the limit of solubility (100 uM) of diazepam in this preparation. RO5-4864 displaced ^{3}H-diazepam binding at similar concentrations. It appears to be a little less potent in the nM range and a little more potent in the uM range. This was typical of three separate experiments.

To explore the biochemical characteristics of the ^{3}H-diazepam binding representatives from various classes of neuroactive agents were studied (table 2). GABA was without effect at 100 nM and 10 uM. The chloride channel antagonists picrotoxinin and pentylenetetrazol were also without effect. Conversely, glycine enhanced ^{3}H-diazepam binding by 50 $\pm$ 14%. Octopamine, 3-isobutyl-1-methyl xanthine, and nifedipine were also without statistically significant effect. The most potent compounds tested were the calcium channel antagonists diltiazem (46 $\pm$ 6.4%) and verapamil (45 $\pm$ 5.7%); the sodium channel antagonists phenytoin (55 $\pm$ 8.5%), carbamazepine (40 $\pm$ 6.4), and lidocaine (52 $\pm$ 4.2%); and the antischistosomal drug praziquantel (51 $\pm$ 14%). The displacements by phenytoin and verapamil were not additive. None of the pesticides except chlordimeform, statistically significantly displaced ^{3}H-diazepam (Table 3).

To further characterize the CNS/peripheral nature of these

TABLE 2

Reduction of ^{3}H-Diazepam Specific Binding to Membranes from the Head of American Cockroach by Various Neuroactive Agents

Agent	Concentration (uM)	Reduction, % of Control [a]	
Diazepam	100	100[b]	A
GABA	10	8.5 ± 20	B C
GABA	0.1	-8.0 ± 4.2	B
Picrotoxinin	100	-11 ± 7.1	B
Pentylenetetrazol	100	-8.5 ± 4.9	B
Glycine	0.1	-50 ± 14	D
Octopamine [c]	100	22 ± 8.5	C E
3-isobutyl-1-methyl xanthine	100	13.5 ± .01	C
Nifedipine	100	24 ± 9.9	C E F
Diltiazem	100	46 ± 6.4	E F
Verapamil	100	45 ± 5.7	E F
Phenytoin	100	55 ± 8.5	F
Carbamazepine	100	40 ± 6.4	E F
Lidocaine	1000	52 ± 4.2	E F
Phenytoin plus Verapamil	100 100	53 ± 1.1	E F
Praziquantel	100	51 ± 14	E F

a Each mean is of two separate experiments performed in triplicate ± standard deviation. Values followed by the same letter are not significantly different by Student Newman Keal multiple comparison procedure at $p < .05$.

b Normalized control.

c Assays terminated at 20 minutes.

TABLE 3

Reduction of ^{3}H-Diazepam Specific Binding to membranes from the Head of American Cockroach by Various Pesticides

Pesticide	Concentration (uM)	Reduction, % of control [a]	
Diazepam	100	100 [b]	A
DDT	100	0 ± 27	B
DDT	10	-6.6 ±	B
Cis-permethrin	100	4.5 ±	B
Trans-permethrin	100	2.4 ± 19	B
Cypermethrin	100	-20 ± 57	B
Decamethrin	1	26 ± 1.5	B
Decamethrin	.01	-16 ± 33	B
Baygon	100	10 ± 15	B
Lindane[c]	100	44 ± 24	B
Guthion	100	25 ± 9.9	B
Nicotine	100	36 ± 13	B
Chlordimeform	100	52 ± 17	C

a Each mean is of two or three separate exeperiments performed in triplicate ± standard deviation. Values followed by the same letter are not significantly different by Student Newman Keal multiple comparison at $p < .05$.

b Normalized control.

c Gamma-hexachlorocyclohexane.

interactions, chlordimeform was tested in rabbit brain and kidney preparations (Table 4). Chlordimeform was moderately potent in the kidney preparation (35 ± 12%), but had no effect in the brain preparation.

Phosphonucleotides are often involved in kinase mediated receptor-message transuction. The nucleotides N^6,2-O-dibutyryladenosine 3':5'-cyclicmonophosphate (dibut-C-AMP), N^2,2'-O-dibutytylguanosine 3':5'-cyclicmonophosphate (dibut-C-GMP) guanosine-5'-diphosphate (GDP), and guanosine-5'-triphosphate (GTP) were tested (Figure 3). Only the guanosine cyclicmonophosphate analog had a significant effect. It increased specific binding by 50 ± 12%.

To determine the proteinaceous nature of the interactive sites of the binding, various denaturation procedures were used (Figure 4). Treatments with sodium dodecyl sulfate (1% w/v), heating at 90^{o} C for 5 minutes, or bovine serum albumin (1% w/v) reduced specific binding by 95 ± 18%, 66 ± 38%, and 36 ± 14%, respectively. The results of trypsin (1% w/v) was variable and the specific binding was not significant. On the other hand, milder treatments of the same agents or methods: sodium dodecyl sulfate (0.1% w/v); trypsin (0.01% w/v); bovine serum albumin (0.01% w/v); and heating the preparation to 90^{o} C increased the specific binding by 64 ± 38%, 51 ± 24%, 61 ± 24%, and 141 ± 20%, respectively.

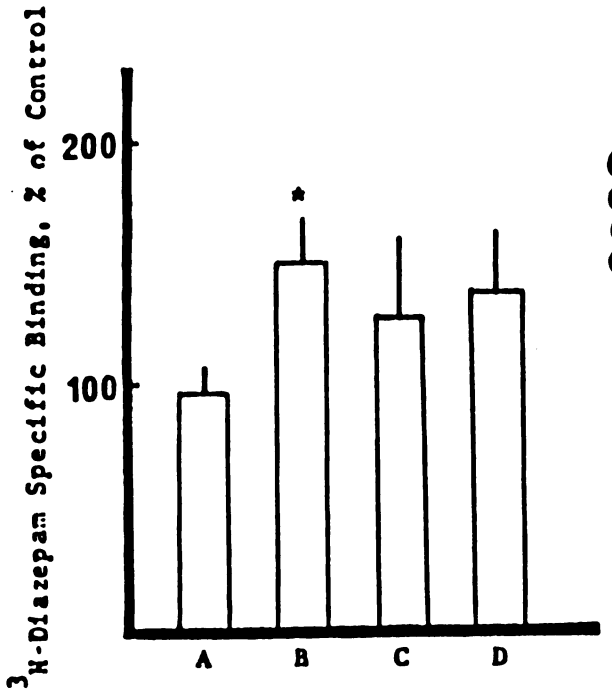
TABLE 4

Reduction of ^{3}H-Diazepam specific binding by 100 uM Chlordimeform to membranes from rabbit brain and kidney

Tissue	Reduction, % of Control [a]
Brain	8.8 ± 7.7
Kidney	35 ± 12[b]

a Each mean is of three separate experiments performed in triplicate ± standard deviation. Control specific binding was determined with 100 uM RO 5-4864.

b $p < .05$ by Ranked Sum Test.

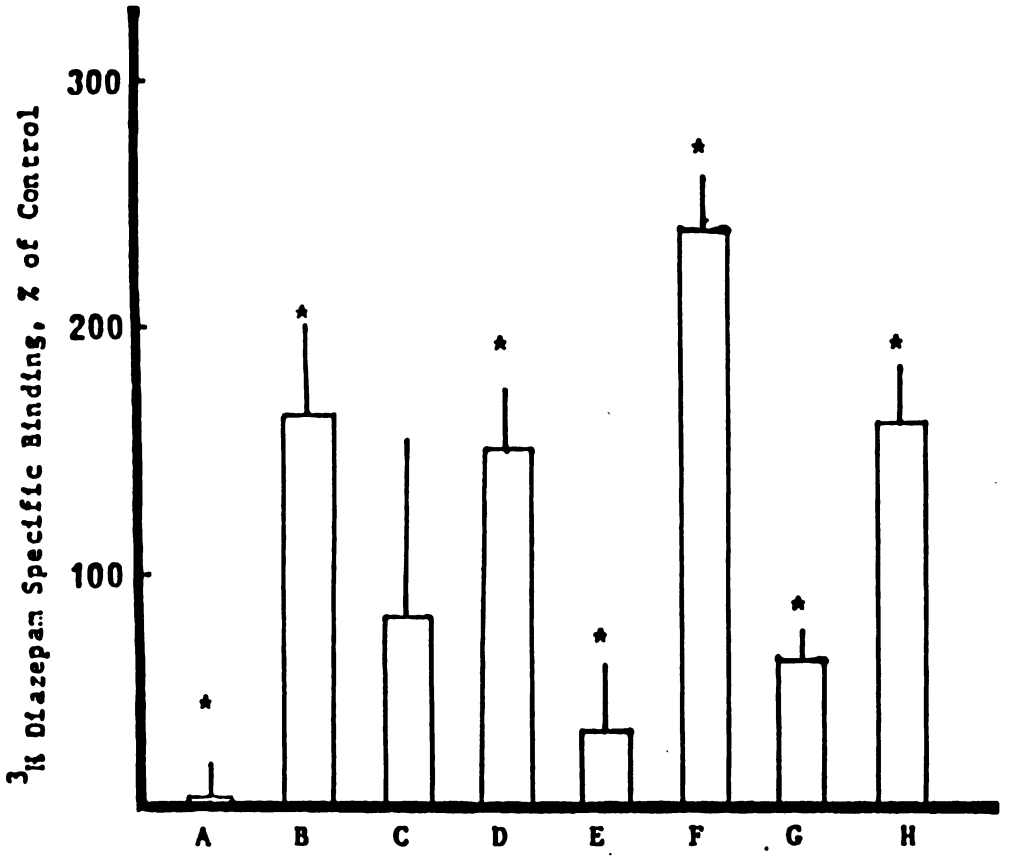


(A) N^6,2-O-dibutyryladenosine 3':5'-cyclic monophosphate
(B) N^2,2-O-dibutyrylguanosine 3':5'-cyclic monophosphate
(C) Guanosine-5'-diphosphate
(D) Guanosine-5'-triphosphate

Figure 3. Effect of nucleotide phosphates (1 uM) on ^{3}H-diazepam (3.6 nM) specific binding. The nucleotides were added 40 minutes after the competitative binding was initiated. The assay was terminated one minute after the addition of the nucleotide. Each bar and line is the mean and standard deviation of one experiment performed in triplicate. (*) .01 <p< .05

(*) .01 <p< .05 by Ranked Sum Test

Figure 4. Effect of denaturation on ^{3}H-diazepam (3.6 nM) specific binding. The isolation buffer was replaced with 0.25 M sucrose. Sodium dodecyl sulfate (A,B) was added to the membranes at 25°C, then placed on ice. Trypsin (12,700 units/mg) (C,D) was incubated for 1 hour at 25°C with the membranes prior to initiating the assay, then placed on ice. Membranes were heated to 90°C in 5 minutes (E,F). Then maintained at 90° for 5 minutes (F only) at 90°C for 5 minutes, then placed on ice. Bovine serum albumin (G,H) was incubated with the membranes for 1 hour at 25°C prior to initiating the assay, then placed on ice. Each bar and line is the mean and standard deviation of one experiment performed in triplicate. (*) $.01 < p < .05$ by Ranked Sum Test.



(A) Sodium dodecyl sulfate, 1% w/v
(B) Sodium dodecyl sulfate, 0.1% w/v
(C) Trypsin, 1% w/v
(D) Trypsin, 0.01% w/v
(E) Heat at 90° C for 5 min.
(F) Heat to 90° C
(G) Bovine Serum Albumin, 1% w/v
(H) Bovine Serum Albumin, 0.01% w/v

Figure 4.

The effects of various ions on ^{3}H-diazepam specific binding was studied in the presence and absence of the calcium chelator ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) (Figure 5). specific binding in the controls of EGTA treated preparations was typically 3-4 times that of the non EGTA controls. $CaCl_2$ added after this treatment did not reverse this increase. All of the salts were added to give a 200 mM cation final concentration except $CaCl_2$ which was tested at 10 mM. All of the salts tested, except $CaCl_2$, increased the specific binding to EGTA treated membranes: NaCl, 70 $\pm$ 315; KCl, 94 $\pm$ 27%; NH_4NO_3, 163 $\pm$ 71%; $(NH_4)_2SO_4$, 215 $\pm$ 45%; Na_2SO_4, 239 $\pm$ 73%; and choline chloride, 143 $\pm$ 71%. $CaCl_2$ enhanced specific binding in the absence of the EGTA treatment by 31 $\pm$ 26%. No other salts enhanced specific binding in the absence of the EGTA treatment. Sucrose (200 mM) decreased specific binding in the non EGTA treated membranes by 51 $\pm$ 18%.

Figure 5. Effect of ions on ^{3}H-diazepam (3.6 nM) specific binding to EGTA treated membranes. Final cation concentration were 10 nM for $CaCl_2$ and 200 mM for all others. Sucrose concentration was 200 mM. Van Harrevelds saline was replaced with 0.25 M sucrose. The final membrane suspension was made in 5 mM Tris-HCl (pH, 7.1) plus 2 mM EGTA. Specific binding to untreated controls was typically 2000-5000 DPM. Specific binding to treated controls was 10,000-15,000 DPM. Specific binding enhancement by NaCl and KCl were concentration dependant. Each bar and line is the mean and standard deviation of one experiment performed in triplicate. (*) ($.01 < p < .05$) by Ranked Sum Test.

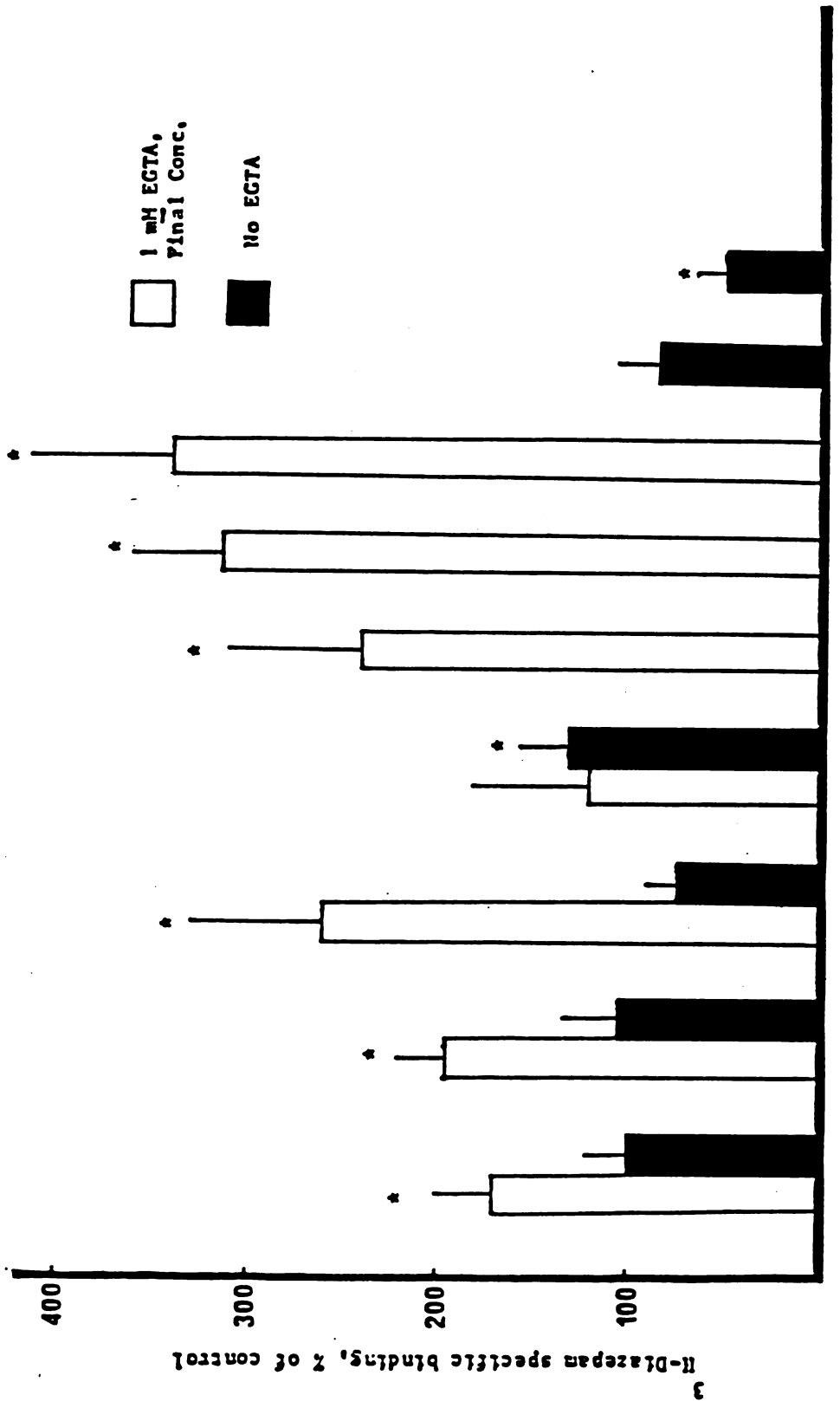

1 mM EGTA, Final Conc.
No EGTA
400
300
200
100
³H-Diazepam specific binding, % of control
NaCl
KCl
NH_4NO_3
$CaCl_2$
Choline Cl^-
$(NH_4)_2SO_4$
Na_2SO_4
Tris- SO_4 Sucrose (pH, 7.1)

Figure 5.

DISCUSSION

^{3}H-diazepam binding to American cockroach head membranes, like that of ^{3}H-flunitrazepam binding to house fly thoracic membranes, is "peripheral". ^{3}H-diazepam specific binding was predominantly to non CNS components of the American cockroach head. R 05-4864 was the most potent benzodiazepine tested in this system, while clonazepam was relatively weak. K_i values for clonazepam and R 05-4864 at the mammalian high affinity "CNS type" receptor (^{3}H-diazepam) are 5nM and 163 uM respectively (8). K_i values at the mammalian low affinity site are 182 uM and 491 uM respectively (5). IC_{50} values for the mammalian peripheral sites range from 2.9 - 7.9 uM and 4.1 nM respectively (6). This difference at the peripheral site is consistent when ^{3}H-R 05-4864 is used as the radioligand (35). Chlordimeform displaces ^{3}H-diazepam from American cockroach head membranes and rabbit kidney membranes, but not rabbit brain membranes. GABA is without effect on ^{3}H-diazepam binding to american cockroach head membranes or to ^{3}H-R 05-4864 binding to rat brain membranes (7), despite the fact that GABA enhances ^{3}H-diazepam binding to rat brain membranes (36). That GABA does enhance ^{3}H-flunitrazepam binding to house fly thoracic membranes may be indicative of a more evolutionarily advanced association of benzodiazepine receptors and the GABA system in the housefly. The significance of glycine in cockroach neurobiology is not yet known.

^{3}H-diazepam binding to American cockroach head membranes also resembles the mammalian low affinity receptor (5). Both have quite low affinity

for the benzodiazepines in general. The mammalian low affinity receptor required unlabeled diazepam in excess of 1 m$\underline{M}$ to achieve saturation. It is conceivable that the American cockroach system could saturate if the solubility of diazepam were greater in this preparation. Phenytoin is quite potent at displacing ^{3}H-diazepam from the mammalian low affinity receptor. The mammalian low affinity receptor has been shown to be associated with a calcium-calmodulin dependent protein kinase that is probably involved in neurotransmitter release (see 37 for review). Diazepam (38) and phenytoin (37) inhibit the kinase thus reducing transmitter release. This might explain, in part, the actions of diazepam on type II pyrethroid toxicity (28).

DDT and pyrethroids alter sodium channel kinetics allowing for an increase in sodium infux (39). The subsequent membrane depolorization would increase the calcium concentration in the presynaptic terminal. The increased transmitter release would enhance the convulsive state. Blocking the transmitter release would help to control it. However, this does not explain the lack of effect of diazepam on the DDT and type I pyrethroid symptoms (28). It is also difficult to reconcile the lower binding to the CNS components of the head if the binding is synaptic only.

R05-4864 has a calcium channel antagonistic effect on the guinea pig heart (40). Nifedipine, diltiazem, and verapamil are all pharmacologically potent calcium channel antagonists, however, nifedipine is thought to act at a site separate but allosterically coupled to the sites for diltiazem and verapamil (41). This would explain nifedipines weaker action on ^{3}H-diazepam binding to the

American cockroach head membranes. Praziquantel exerts at least part of its antischistosomal activity by opening or blocking open calcium channels (42-44). A calcium channel interaction is favorable in light of the higher ^{3}H-diazepam binding to the muscular components of the cockroach head. It could also explain the lack of movement seen in diazepam intoxicated German cockroaches (32). Again it does not adequately explain the selective inhibition of type II pyrethroids.

Verapamil (45) and its methoxy derivative D600 (45-47) block voltage sensitive sodium channels when applied in u<u>M</u> concentrations. Veratridine and batrachotoxin are neurotoxic alkaloids that activate voltage sensitive sodium channels via the same mechanism (48). D600 and calcium competitively inhibit veratridine activated ^{22}Na uptake in heart cells and neuroblastoma cells (46). Phenytoin, carbamazepine, and diazepam, at u<u>M</u> concentrations, competitively inhibit batrachotoxin activated ^{22}Na uptake and batrachotoxinin A 20-a-benzoate binding in (to) neuroblastoma cells and rat brain synaptosomes (49). Lidocaine competitively inhibits batrachotoxinin A 20-a-benzoate binding to rat brain synaptosomes at a concentration of 1m<u>M</u> (50). That phenytoin and verapamil displacement of ^{3}H-diazepam is not additive supports the idea that these compounds are acting at the same site. Flurazepam, at high u<u>M</u> concentrations, reduces sodium and potassium activation currents in nerve fibers of the frog <u>Rana esculenta</u> (51). Chlordimeform reduces sodium and potassium activation currents in crayfish giant axon at high u<u>M</u> to m<u>M</u> concentrations (52). Unlike flurazepam or local anesthetics, chlordimeform also produces a sodium inactivation tail current that leads to repetitive firing of the axon. This

repetitive firing has also been observed in the American cockroach central nerve cord and is blocked by elevated calcium in the saline bath (53).

Calcium is also important to the ^{3}H-diazepam binding site from the American cockroach head. Chelation could cause a denaturing of the binding site or the dissociation of some component of the site in the membrance. How the various ions enhance this effect is not known yet. That the effect of EGTA is not reversed by the addition of calcium supports the idea that this effect is more than simple deprivation of calcium from the binding site. Mild denaturation procedures also enhance the binding, lending further support. Stronger treatments denature the binding site sufficiently to reduce specific binding. This indicates a proteinaceous nature to the binding site. Bovine serum albumin should have no denaturing ability of its own. It may be chelating calcium and allowing the conformational change at the lower concentration. At the higher concentration it may simply be out competing the membranes for the diazepam, since it was in a 10-20 fold excess compared to the membrane proteins.

It is interesting that DDT and the pyrethroids did not significantly affect the binding. This indicates that the binding of these pesticides would have to be at different sites of the channel from those ligands that did interact. DDT and type I pyrethroids hold activated sodium channel inactivation much longer, seconds to minutes (39). The diazepam binding site location or kinetics may allow the selective blockade of this longer sodium channel activation.

Octopaminergic systems have been implicated as targets for

chlordimeform actions (54, 55). The American cockroach brain contains an octopamine sensitive adenylate cyclase (56). Diazepam has no effect on ^{3}H-octopamine binding to Drosophila melanogaster head membranes (55) and octopamine has no significant effect on ^{3}H-diazepam binding to American cockroach head membranes. The action of chlordimeform on the diazepam binding site in American cockroach head appears to be independent of an octopaminergic system. Adenylate cyclase systems usually have a regulatory subunit that upon binding GTP reduces the affinity of the receptor and binding to it. Metabolism of GTP to GDP inactivates the cyclase. When GDP dissociates from the regulatory subunit, the receptor reverts to its higher affinity state (57). Phospho diesterase (PDE) metabolizes C-AMP, the product of adenylate cyclase activity. Diazepam (58) and 3-isobutyl-1-menthyl-xanthine (59) are potent PDE inhibitors. That none of these agents affected ^{3}H-diazepam binding indicates that the binding is not associated with an adenylate cyclase coupled, receptor mediated system. The significance of the dibut-C-GMP stimulated binding is not known.

From the data presented here, I conclude that the ^{3}H-diazepam binding site in American cockroach head membranes is probably associated with voltage sensitive sodium channels in the nerve and muscle membranes. Chlordimeform may be exerting part of its toxic actions through this site.

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