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INTERACTION BETWEEN THE BENZODIAZEPINE SYSTEM AND PITUITARY-ADRENAL AXIS

*City University of New York* PH.D. 1983

# INTERACTION BETWEEN THE BENZODIAZEPINE SYSTEM AND PITUITARY-ADRENAL AXIS

by

DORIS M. JACKSON

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

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Abstract

INTERACTION BETWEEN THE BENZODIAZEPINE SYSTEM AND PITUITARY-ADRENAL AXIS

by

Doris M. Jackson

Advisor: Robert L. Thompson

In a biochemical and behavioral study, interaction between the benzodiazepine system and the pituitary-adrenal axis was investigated. Blood-borne substances of 16,000 daltons and 2,000 daltons with brain benzodiazepine binding inhibitory capacity were characterized as heat and acid labile, hydrophilic, and putatively proteinaceous in nature. The 2,000 dalton moiety may represent a fragment of the 16,000 dalton substance. Inhibitory capacity of these serum components was also present in hypophysectomized and adrenalectomized rats. Benzodiazepine receptor densities in the hippocampus, cortex, and cerebellum were unaltered by hypophysectomy and adrenalectomy. It was concluded that neither the pituitary nor the adrenal gland mediate benzodiazepine activity through regulating levels of the putative endogenous ligand or receptor densities in these brain areas.

The benzodiazepine-sensitive, thirsty-rat punish-

ment conflict model of anxiety was used to examine the effects of shock intensity and chlordiazepoxide; and then at a constant shock intensity of 0.2 milliamperes, the effects of $ACTH_{4-10}$, corticosterone, adrenalectomy, and hypophysectomy. Adrenalectomized and hypophysectomized rats were run with and without acute chlordiazepoxide treatment. $ACTH_{4-10}$ effects were also examined against a background of chronic chlordiazepoxide treatment. No significant effect for any hormone or surgical treatment was found except for an anxiolytic effect of $ACTH_{4-10}$ in animals pretreated for five consecutive days with i.p. injections of water. It was tentatively concluded that $ACTH_{4-10}$ which is usually anxiogenic may have an anxiolytic effect in animals sensitized by some pretreatments.

## ACKNOWLEDGEMENTS

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The existence of specific benzodiazepine receptors in certain brain areas gives new direction to investigations concerned with neural substrates of anxiety and the biochemical regulation of such substrates. The benzodiazepines are effective in altering fear-mediated behavior as inferred from their punishment-reducing effect in approach-withdrawal conflict tests. Animals exposed to punishing events and humans suffering from anxiety experience elevations in pituitary-adrenal activity. Hormones from these glands have been posited as active in the central nervous system producing alterations in avoidance behavior. Following sections will discuss and reference these points.

The present study was carried out in order to investigate a possible locus of interaction for the benzodiazepine system and the pituitary-adrenal axis, two systems which appear involved in the effects of punishment. The work was done along two separate lines of research: behavioral and biochemical.

For behavioral investigations an animal model of anxiety was employed. Animal models of anxiety, for the most part, are designed around criteria of drug selectivity. (Lippa, Nash, & Greenblatt, 1979) A thirsty rat conflict test (Vogel, Beer, & Clody, 1971)

was used to establish sensitivity to benzodiazepine and for calibration for sensitivity to anxiogenic as well as anxiolytic agents. Following this, alterations in adrenocorticotropic (ACTH) and corticosterone levels were examined for their ability to affect responding in this test.

On a biochemical level, a partial characterization of an endogenous blood-borne ligand for the benzodiazepine receptors was initiated. This ligand, and the benzodiazepine receptors themselves, were evaluated as possible sites of interaction between benzodiazepine and the pituitary-adrenal axis.

## Animal Models of Anxiety

Several psychoactive agents have the capacity to alleviate anxiety in humans. These include: ethanol, barbiturates, and benzodiazepines. The benzodiazepines diazepam (Valium) and chlordiazepoxide (Librium) are commonly prescribed for their anxiolytic effects. The benzodiazepine class also includes oxazepam, flunitrazepam, clonazepam, and several others. Anxiolytic drugs are identified and examined through animal behavioral tests. These tests constitute an important aspect of preclinical research and are thought to model certain aspects of human functioning. The full range of behavioral effects of antianxiety drugs is reviewed by Gray (1982). The remainder of this section deals with a limited set of paradigms of central interest in evaluating benzodiazepines.

Animal models of anxiety include conflict of passive avoidance type. These tests meet with several important criteria for animal models of a human condition and are useful for identifying and evaluating the effects of anxiolytic drugs. They distinguish side effects from therapeutic effects, they are sensitive enough to detect drug activity at physiologically reasonable doses in the range found effective in humans, the relative potency of various

anxiolytic agents in the tests correlates well with relative potency of therapeutic activity, and tolerance does not develop in the test as it does not with therapeutic administration (Lippa, Greenblatt, & Pelham, 1977).

In general, conflict procedures employ a highly probable response which is suppressed or inhibited by punishment, usually footshock. Administration of certain drugs disinhibits or otherwise interferes with the inhibition of the punished behavior even though the punishment contingency is still present. These tests are referred to as animal models of anxiety because drug-induced disinhibition is peculiar to anxiolytic drugs like barbiturates, ethanol, meprobamate or the benzodiazepines. (Cook & Davidson, 1973; Geller & Seifter, 1960).

Geller and Seifter (1960) reported a procedure with the capacity to detect both the antianxiety and depressant effect of drugs. This procedure utilized a multiple schedule of punished and unpunished responding in which, after several weeks of training, rats showed stable low rates of responding in the punished component and stable high rates in the unpunished component. Drug-induced increases in responding in the punished components were considered to be anxiolytic effects while decreases in responding in

the unpunished component a depressant effect. Several drugs were tested and only those which decreased anxiety in humans like the minor tranquilizers were effective in releasing punished responding while responding in the unpunished component remained unaltered in the same dose range. Neuroleptics and amphetamine both increased suppression.

In a modification of the Geller and Seifter procedure, Davidson and Cook (1969) found similar results. In their procedure a modified multiple variable interval 30-sec, fixed ratio 10 (mult VI 30 FR 10) reinforcement schedule was used. Lever presses were reinforced by food delivery in the unpunished VI component and in the FR component simultaneous food delivery and foot shock occured after every tenth response. In this test barbiturates, benzodiazepines, meprobamate and ethanol were examined and found to dose-dependently release punished behavior but to not affect unpunished behavior. These drugs are all anxiolytic in humans and those which have other psychotherapeutic effects showed a different profile in this test. Chlorpromazine, an antipsychotic agent, depressed both punished and unpunished responding while imiprimine, a tricyclic antidepressant and haloperidol, a dopaminergic receptor blocker, were ineffective. Amphetamine was also tested and it

further suppressed punished response levels at a dose range which left unpunished responding unaffected. An analgesic, morphine, was examined and found not to affect punished responding. This suggests that the action of the anxiolytics was not due to analgesia. Removal of the tone signalling the onset of the punished component of the schedule produced some release from suppression which was only visible after several days, indicating benzodiazepine effects usually seen immediately after drug administration were not produced by interference with stimulus discrimination. In addition, Davidson and Cook found more extreme food deprivation was ineffective in mimicking the effects of anxiolytics, implying food motivation was not a mediating factor in the drug induced disinhibition of punished behavior.

The rank order of potency for the various benzodiazepines in the Cook and Davidson procedure was the same as that found by Geller and associates and was very similar to their relative clinical potency. This was also the case in a reliable and efficient test developed by Vogel et al. (1971) which employs no special training. This test employs a concurrent schedule of continous water reinforcement and FR 20 mouth shock contingent on water spout licking. In the absence of shock, 48-hour water-deprived rats engage in about 700 licks during a 5-minute period after place-

ment in the test chamber. The introduction of shock at 700uA will inhibit licking to about 60-100 licks (limiting shock episodes to 3-5 events). Administration of benzodiazepines disinhibits this licking behavior until the animals will take more than 30 shocks. This test has shown excellent validity for the detection of anxiolytic compounds (Lippa, Nash & Greenblatt, 1979; Vogel et al. 1971).

That the disinhibitory effect of benzodiazepine is not specific to suppression produced by shock punishment was shown by Margulis and Stein (1967) who found it also disinhibited responding during extinction, suppression by punishing brain stimulation or by the taste of quinine. Other researchers have found benzodiazepines effective in releasing exploratory behavior suppressed by novel differentiated (spatial irregularity produced by objects of differential illumination) environments (Crawley & Goodwin, 1980; Hughes, 1972). In a conditioned emotional response (CER) procedure a benzodiazepine increased leverpressing for sucrose solution which had been suppressed by the occurance of a conditioned stimulus in the form of a flashing light which had been previously paired with shock (Guerin, Crenshaw, Smith, Cherek, & (Lane, 1981). Also, Davis (1979) demonstrated that benzodiazepine attenuated acoustic startle amplitude which had been potentiated by the presence of a light

previously paired with shock. Several of these studies show that the disinhibitory effects of benzodiazepines are not specific to the presence of punishment but also occur in the absence of punishment, but in the presence of conditioned stimuli which have been associated with punishment.

File and Hyde (1978) have shown in another kind of test that release of suppression produced by physically noxious stimuli past or present is not the only condition under which benzodiazepines are active. These investigators developed a test where the time spent in active social interaction between pairs of male rats is monitored. Social interaction behavior is monitored under different conditions of familiarity with the test chamber and different levels of illumination. Increasing degrees of anxiety-inducing conditions are considered to be represented by: familiarity with the test box under low light conditions, familiarity under high light intensity, unfamiliarity during low light intensity, and unfamiliarity with high light levels. Each animal is tested in only one condition and averaged results show a significant drop from 600 seconds to 250 seconds of social interaction time across these four increasingly anxiety-provoking conditions. After treatment with chlordiazepoxide, a benzodiazepine, this drop was completely eliminated. Interaction time remained high

even in the highest anxiety condition. General activity levels were not altered by either drug or test condition so it was concluded that drug and test effects were on anxiety.

The pharmacological activity of anxiolytic agents commonly examined in conflict tests can also be tested for the capacity to inhibit pentylenetetrazole-induced convulsions. Intravenous injection of pentylenetetrazole results in seizures followed by death. Prior administration of benzodiazepines inhibits these seizures with a relative potency correlated with relative potency in conflict tests.

In addition to the correlations between relative potencies of the various benzodiazepines for relieving anxiety in humans and disinhibiting punished behavior and antagonizing pentylenetetrazol-induced seizures noted in animals, there is an important biochemical correlate between relative potency in all of these tests and relative potency in the capacity of the benzodiazepines to stereospecifically bind, with high affinity, to receptors contained in synaptosomal membranes prepared from rat brain tissue (Lippa, Klepner, Sano, Yunger, Smith, & Beer, 1978; Mohler & Okada, 1977(b); Squires & Braestrup, 1977). These receptors were identified through the use of radioreceptor assays.

## Radioreceptor Assays

### Basic Principles

One of the ways which drugs may produce their effects is through a capacity to bind to specific receptor sites in biological tissues. These drugs constitute a ligand for the receptor site. Ligands may be naturally occuring as well as synthetically produced. For example, the analgesic morphine has been found to act as a ligand for a binding site which also interacts with a naturally occuring ligand, endorphin. Ligands can be labeled with radioactive material and used in radioreceptor assays for the analysis of the sites of action of drugs.

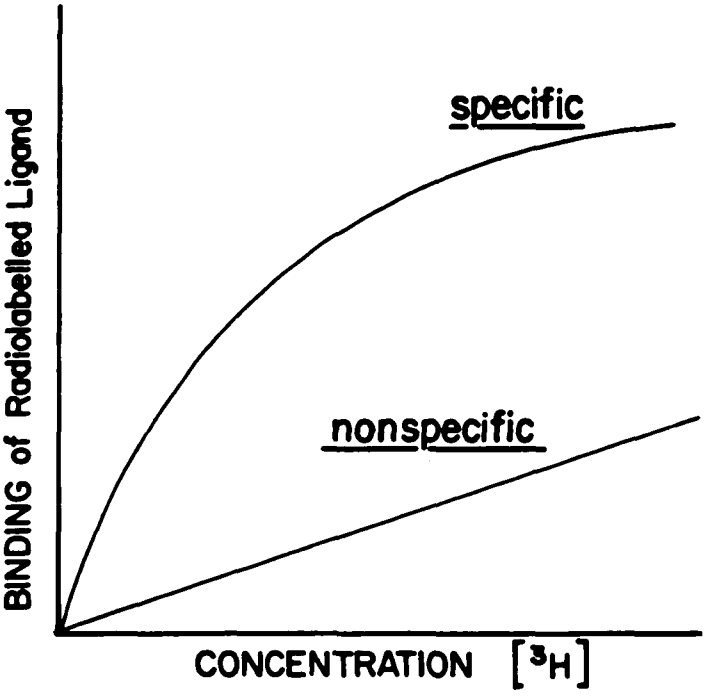
Use of radiolabeled ligands permits quantitative experimentation concerning numbers of binding sites and strength of the binding tendency or affinity of the ligand for the site. Incubation of high-affinity radiolabeled ligands with receptor-containing tissues results in saturable, specific binding to the receptors in addition to unsaturable nonspecific binding.

All bound radiolabeled material will remain on the tissue after unbound radiolabeled ligand is removed by filtration of centrifugation. Saturability of binding indicates the presence of a finite number of receptors and is part of the criterion for receptor identification.

The saturable or receptor-specific binding is determined by use of two parallel sets of assay tubes. One set contains receptor-contraining tissue and radiolabeled (tritiated) ligand. The second set contains both of these and in addition a large amount of untritiated ligand of the same kind. Binding which occurs in the presence of the untritiated ligand is considered nonspecific binding since the presence of a high concentration of untritiated ligand entirely displaces the tritiated ligand from specific binding sites while not affecting nonspecific binding. Specific binding is the total binding minus the nonspecific binding or the amount of binding which occurs in the tubes without the unlabeled displacer minus that which occurs in the tubes with the displacer.

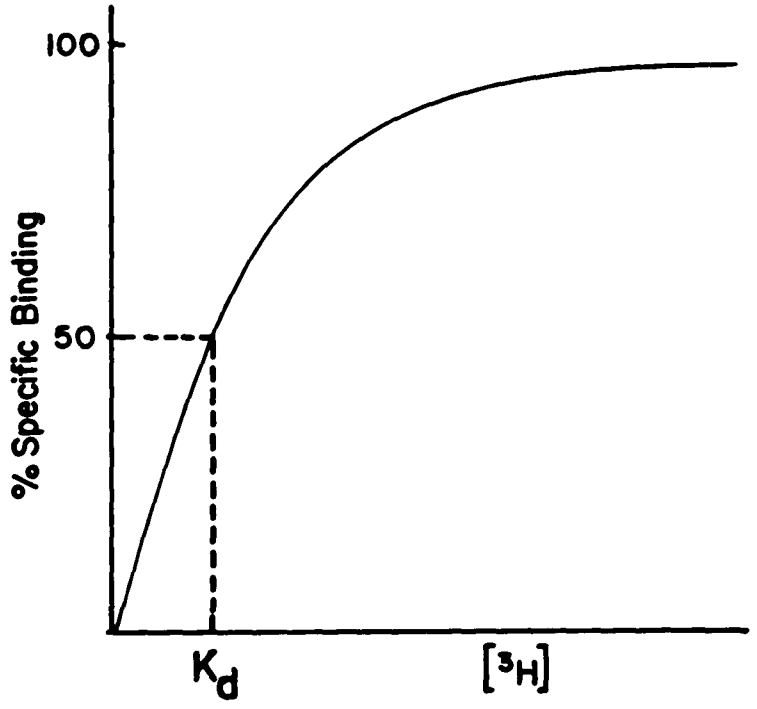
With increasing concentrations of radiolabeled ligand both specific and nonspecific binding will increase. Specific binding levels off as larger and

larger proportions of the limited number of binding sites are occupied while nonspecific binding will continue to increase. The first is termed saturable binding and is by accepted definition considered to be specific binding and the latter unsaturable binding is termed nonspecific binding.

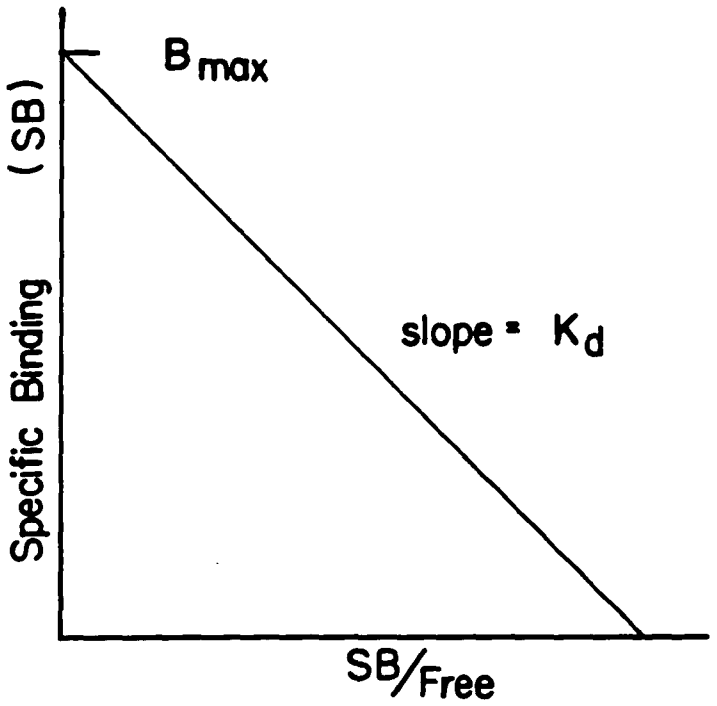


Saturation-Isotherm

Specific binding is a function of the number of receptors present ($B_{max}$) and the affinity of the ligand for the receptor ($K_d$). These can be measured by employing varying concentrations of radioactive ligand from a high saturating concentration to much lower concentrations. The degree of binding will be asymptotic at high concentrations. This represents the maximal amount of binding possible to a finite number of receptors, $B_{max}$, or saturability.



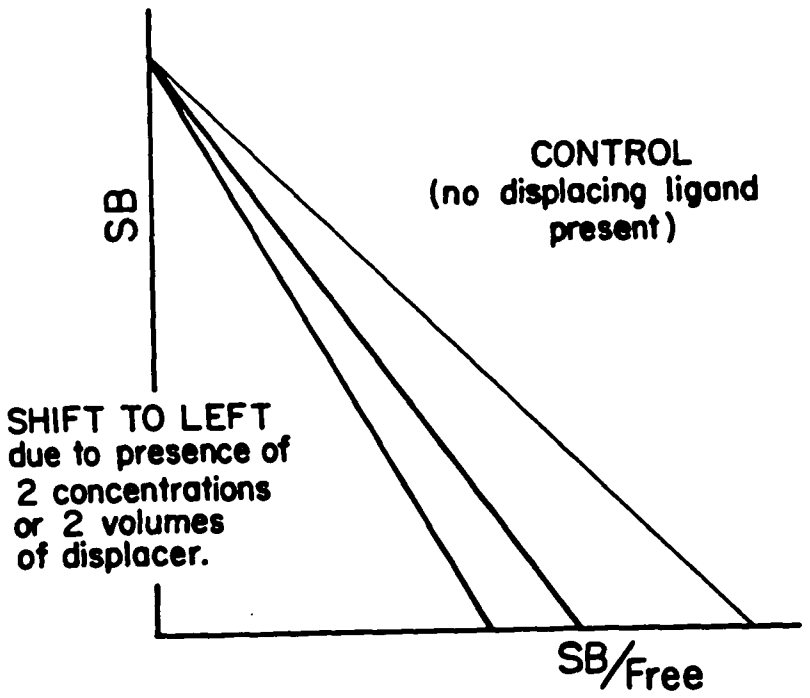
The concentration of radioligand which produces 50% of the maximal specific binding is termed the $K_d$ value and is an indication of affinity of the ligand for the receptor. High affinity is represented by a low $K_d$ and low affinity by a high $K_d$ since a greater concentration of ligand would be necessary for occupation of 50% of the binding sites. $K_d$ values are also obtained by the slope of the line in a Scatchard plot. In this plot specific binding is plotted against the ratio of specifically bound labeled ligand (SB) to available but unbound (free) ligand or, in other words, the proportion of available ligand which is bound at different concentrations of ligand/tube.



$B_{max}$ may also be obtained from the Scatchard plot by extrapolating from a linear relationship between specific binding and bound/free to a value commensurate with saturating concentrations or maximal binding.

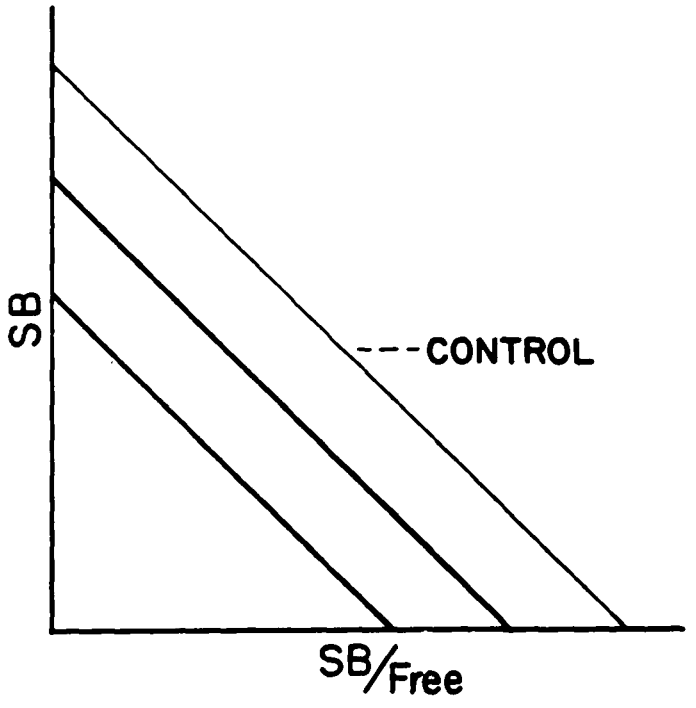
Displacement

Various ligands may inhibit specific binding by different mechanisms. One means of studying these mechanisms involves determining Scatchard plots in the absence and presence of various concentrations of an unlabeled displacer. An active inhibitor of specific binding will alter the curve of tritiated ligand bound. This alteration may occur through a change in slope indicating a change in affinity ($K_d$) of the



labeled ligand for the receptor. This $K_d$ shift with no alteration in $B_{max}$ has been termed competitive inhibition.

Displacement may also be characterized by a lowering of $B_{max}$ where binding of the unlabeled ligand is not reversible and can not be overcome by high concentrations of the high affinity labeled ligand. This type of binding is termed noncompetitive.



A mixed form of inhibition may also occur where a shift in $K_d$ along with a lowering of $B_{max}$ is seen.

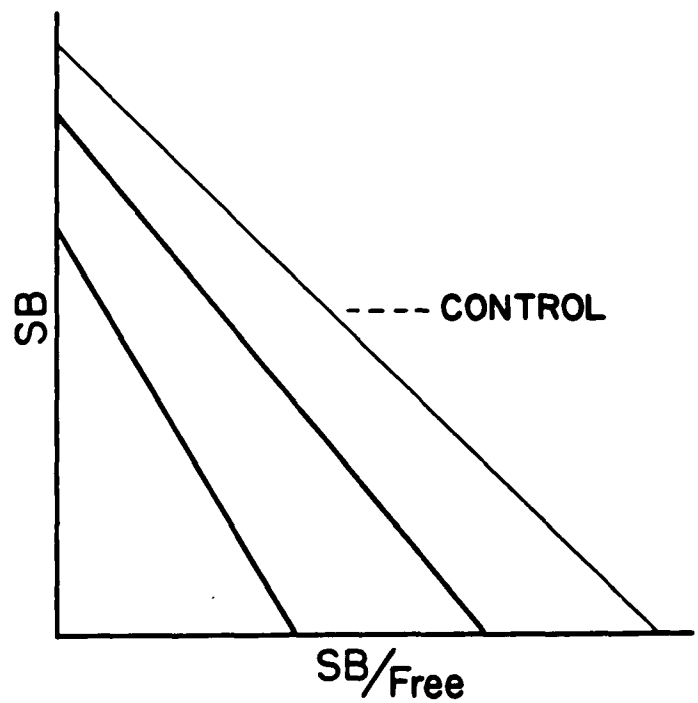



Figure F

For extensive detailed descriptions of radioreceptor binding and interpretation of data derived from these assays see Bennett (1981) and Burt (1981).

## Benzodiazepine Receptors

The presence of specific benzodiazepine receptors in the central nervous system is now well established. Binding studies reveal high densities of saturable, stereospecific binding sites in the cortex, hypothalamus, cerebellum, midbrain, and hippocampus (Mohler & Okada, 1977a; Squires & Braestrup, 1977). The various benzodiazepines bind to these receptors with different $K_d$ values which correlate with their potency in disinhibiting punished behavior and also in tests that measure the anticonvulsant and muscle relaxant effects of the benzodiazepines (Braestrup & Squires, 1978; Mohler & Okada, 1977b).

Peripheral binding sites have been found in tissues prepared from platelets, kidney, heart, lung and retina (Braestrup & Squires, 1977; Howells, Miller, & Simon, 1979; Taniguchi, Wang, & Spector, 1981; Wang, Tanaguchi, & Spector, 1980). The relative $K_d$ values of the benzodiazepines at the peripheral sites differ from those at central binding sites with some very potent at one while ineffective at the other (Braestrup & Squires, 1977). For example, the affinity of flunitrazepam for frontal cortex synaptosomal membranes is 10 times greater than that displayed for kidney and R05 4864 has high affinity for kidney, but nearly none for brain.

(Braestrup, & Squires, 1977) (See also this manuscript, Experiment 9 in the Biochemistry Results section).

Through the use of lesioning, cell deterioration and cell culture studies central binding sites have been localized as residing on neural and glial cells rather than on other tissues (Baraldi, Guidotti, Schwartz, & Costa, 1979; Braestrup, & Squires, 1978; Bresolin, Speth, McManus, Yamamura, & Stern, in press; Meyerson, Sano, Critchett, Beer, & Lippa, 1981). Localization to synaptosomal membrane fractions has been demonstrated by Bosman, Penney, Case and Averill (1980) and DeRobertis, Alberici, Rodriguez es Lores Arnaz, and Agrurra, (1967).

Receptor Plasticity

Increases and decreases in the number of neurotransmitter receptors appear to be a homeostatic mechanism in several transmitter systems. In general, decreases in brain binding sites are seen after treatments which increase receptor stimulation while increases in binding sites result from a decrease in receptor stimulation. Several examples are: up-regulation of dopaminergic and adrenergic receptors after denervation, up-regulation of dopaminergic, adrenergic and muscarinic receptors after chronic

treatment with receptor blockers (Ben Barak, Gazit, Silman & Dudai, 1981; Ebstein, Pickholtz, & Belmaker, 1979; Hitri, Weiner, Borison, Diamond, Nausleda, & Klawans, 1978; McKinney, & Coyle, 1982) and down-regulation of dopaminergic and muscarinic receptors after chronic administration of agonists (Ben Barak et al., 1981; McKinney & Coyle, 1981). Decreases in benzodiazepine binding sites are seen after chronic treatment with flurazepam (Chiu & Rosenberg, 1978). Also other experimental manipulations produce alterations in benzodiazepine binding sites. Of particular interest here is that increases and decreases in benzodiazepine binding site densities can be seen after stress-inducing manipulations of different types.

Increases in binding are seen after immobilization stress, cold water swim, and following seizures (Braestrup, Nielson, Nielson, & Lyon, 1979; Paul & Skolnick, 1978; Soubrie, Thiebot, Jobert, Montastrue, Hery, & Hamon, 1980). Lippa et al., (1978) showed that electric footshock decreased $^{3}$H-diazepam binding by 14%. A greater decrease was seen in animals exposed to a conflict procedure utilizing foot shock. Even though both groups received the same number of shocks the group exposed to the approach-avoidance conflict situation in addition to exposure to foot shock showed significantly greater decreases in

binding. A 25% decrease in binding has also been seen after presentation of a tone previously paired with shock, representing a greater decrease in binding than seen in animals receiving tone alone (Guerin et al., 1981). These findings are particularly interesting because receptor changes were greatest when examined after the occurence of a stimulus not itself physically noxious but with a history of association with a physically noxious event. This suggests a neural substrate more sensitive to anxiety than to pain may be involved with these receptors.

Since increases and decreases have been seen following stressful treatments we may conclude that the relationship between stress and receptor changes is not a qualitatively simple one. There is a need for studying alterations in the benzodiazepine receptors with respect to the qualitative and quantitative characteristics of particular stressing situations. The suspicion of several researchers cited throughout this paper is that stress is far from a unitary phenomenon and may have different physiological consequences possibly concerning its physical or psychological nature, its intensity, or perhaps with discriminability of the stressing stimulus or time of occurance, etc. These considerations have been incompletely dealt with under the rubric of discriminating

fear from anxiety but more intensive experimentation is needed using stress characteristics as independent variables.

## Endogenous Ligands

### Methods for Isolation and Characterization

The existence of benzodiazepine-specific receptors in synaptosomal membrane fractions isolated from the brains of all vertebrates tested except the cartilagenous fish has lead to investigations designed to reveal the presence of endogenous ligands for these receptors (Braestrup & Squires, 1978; Mohler & Okada, 1977(a); Mohler & Okada, 1977(b); Squires & Braestrup, 1977). By investigating the capacity for $^{3}$H-benzodiazepine displacement in receptor binding assays, several physiological tissues such as brain, cerebrospinal fluid, and intestine appear to contain such a ligand (Davis & Cohen, 1980; Guidotti, Toffano, & Costa, 1978; Kuhn, Neuser, & Prunztek, 1981; Massoti, Guidotti, & Costa, 1981; Woolf & Nixon, 1981). The general methods of investigation employed involve homogenization of tissue in buffer, centrifugation to remove high density components and particulate matter, and subsequent assay for displacing capacity. If the tissue homogenate proves active in the assay, chro-

matography is usually carried out in order to purify, characterize, and identify, if possible, the active constituent.

Gel filtration has been extensively utilized as a method of fractionation and purification. Receptor binding assays carried out on periodically collected samples emerging from the filtration column determines the elution time of the active component. This elution time can then be compared to the elution time of standards of known molecular weight for determination of molecular weight of the active constituent. A faster and very accurate technique for separation by molecular weight is high performance liquid chromatography (HPLC) (Hashimoto, Sasaki, Aiura, & Kato, 1978). This rapid pressurized processing of a sample through an appropriately packed column can be done in less than 1 hour depending upon the volume of the sample and can employ both aqueous and organic mobile phases. Periodically collected samples of the eluate can then be assayed for activity and elution time compared to that of standards of known molecular weight.

Relative hydrophobicity/hydrophilicity of active substances is frequently examined. This determination can be carried out with ammonium sulphate precipitation or by use of SEPAK reverse phase cartridges

(Gay & Lahti, 1981), or other techniques. Other properties examined include heat and acid stability.

## Endogenous Ligand Candidates for the Benzodiazepine Receptor

Skolnick, Marangos, Goodwin, Edwards, and Paul (1978) and Marangos, Clark, Martino, Paul, and Skolnick (1979) describe an extract of bovine brain capable of competitively inhibiting diazepam binding from rat brain membranes. Inhibiting substances were identified as the purines, inosine, and hypoxanthine, by several chromatographic methods. The affinity of these purines was found to be much lower (mM range concentrations of purines are necessary for measurable binding) than that of diazepam (nM range). But, it is argued by these researchers that since 10-20% receptor occupation is pharmacologically relevant this low affinity does not rule out the purines as a legitimate endogenous ligand. (Lippa, Critchett, Sano, Klepner, Greenblatt, Coupet, & Beer, 1979). Purine candidacy is further supported by the synthesis of a triazolopyridazine, a bio-isosteric to the purines, and demonstrating nM range receptor affinity and pharmacological efficacy apparently more specific for the anxiolytic effect than diazepam which produces, in addition, muscle relaxant and sedative effects upon acute administration.

GABA modulin has also been presented as a possible natural benzodiazepine receptor ligand (Guidotti, Toffano, & Costa, 1978). This 15,000 dalton peptide interferes with the facilitation of GABA binding usually seen in the presence of benzodiazepines.

Massotti, Guidotti, and Costa, (1981) also have recently reported a heat-stable, protease-degradable entity extracted from brain tissue which is able to inhibit benzodiazepine binding. A displacing factor of 40,000-70,000 daltons has been reported by Colello, Hockenberry, Bosman, Fuchs and Folker (1978) in addition to a smaller fragment of one-fifth potency. Another inhibitor of benzodiazepine binding of about 3,000 daltons has been identified by Davis and Cohen (1980). This substance isolated from bovine brain is heat-stable and degradable by papain, suggesting a peptide nature. In addition, anticonflict and EEG activity following intraventricular application of this extract show diazepam-like alterations.

Multiple endogenous displacers of $^{3}$H-diazepam have been found in human cerebrospinal fluid (Kuhn, Neuser, & Prunztek, 1981). Chromatographic treatment revealed two major peaks of activity which were resolved as having a molecular weight between 700-3600 daltons. Another peak of activity was found at

greater than 10,000 daltons molecular weight. Inhibitory activity of the smaller two was GABA enhanceable which was not the case for the larger component.

A protein has been isolated from bile duct and small intestine of rats which inhibits the binding of $^{3}$H-diazepam from brain binding sites (Woolf & Nixon, 1981). This competitive inhibitor has a molecular weight of about 16,000 daltons and when treated with pronases yields a smaller fragment retaining activity.

Many laboratories have had positive findings using varying techniques to examine diverse tissues. Many may have happened upon the same ligand from different approaches, since alteration of characteristics by biochemical treatments may produce an apparent but not actual multiplicity of ligands being discovered by different researchers. On the other hand, several ligands are likely to exist and researchers have undoubtedly discovered several of these.

## Pituitary-Adrenal Axis and Anxiety

### Stress Sensitivity and Avoidance

Selye's (1956) description of the general adaptation syndrome emphasized the role of the pituitary-

adrenal (P-A) axis in organismic adaptation to stress. He described an immediate alarm reaction upon exposure to a stressor, such as electric shock, consisting of a rise of adrenocorticotrophic hormone (ACTH) levels within 30 sec. This elicits and is followed by a rise in glucocorticoid secretion within 3-5 min which lasts as long as the stressor is present or until adrenal "exhaustion". Other researchers have found modifications in levels of P-A hormones as a result of stress or emotional experiences.

Mason (1968) examined the alterations in blood levels of several hormones before, during and after experience with a Sidman avoidance schedule and found a tripling of corticosterone secretion during the entire avoidance period which returned to normal several days after termination of the avoidance period.

Avoidance behavior has also been shown to be positively correlated with circadian rythms of secretion of P-A hormones. Both are higher at night than in the early morning. Positive correlations between glucocorticoid levels and avoidance behavior have been extensively demonstrated (Gold & Van Buskirk, 1976; Pagano & Lovely, 1972). Time course of post stress diminishing glucorticoid levels in blood and extinction of avoidance behavior correspond (Levine & Brush,

1967). Also, individual rats which show greater relative glucocorticoid responsiveness to stress show more efficient Sidman avoidance relative to individuals with more attenuated glucocorticoid responsiveness (Wertheim, Conner, & Levine, 1969).

Similar behavioral changes can be produced through alterations of hormone levels by surgery or by injection. Removal of the pituitary gland reduces levels of P-A hormones and hypophysectomized animals show reduced ability to acquire active and passive avoidance responses and increased rates of extinction (Appelzweig & Baudry, 1955; Weiss, McEwen, Silva & Kalkut, 1970). Replacement therapy with ACTH restores behavior to normal due to either ACTH itself or increased levels of glucocorticoids stimulated by ACTH.

ACTH injections, which increase both ACTH and glucocorticoids levels, administered to intact animals appear to retard extinction of avoidance behavior in a dose dependent manner (DeWeid, 1969; Levine & Jones, 1965; Miller & Ogawa, 1962; Murphy & Miller, 1955) while effects of ACTH administration on acquisition are mixed. (Murphy & Miller, 1955). Adrenalectomized animals with high circulating levels of ACTH and low levels of glucocorticoids have been employed to separate the effects of ACTH and the glucocorticoids. In general, the adrenalectomized

animals behave like ACTH-treated animals with respect to avoidance even though the levels of glucocorticoids are very low. Hence, demonstrated behavioral changes subsequent to raised levels of ACTH appear to be due principally to ACTH itself rather than to ACTH-induced elevations in glucorticoids (Beatty, Beatty, Bowman, & Gilchrist, 1970; Silva, 1974; Weiss, McEwen, Silva, & Kalkut, 1969).

In contrast, glucocorticoid administration to intact rats, producing high circulating levels of glucocorticoids along with low ACTH levels due to negative feedback, results in retarded acquisition and accelerated extinction of avoidance responding (Endroozi & Fekete, 1973; Levine & Levin, 1970; Wimersma Greidamus, 1970).

## P-A Axis and Psychological Stress

In the studies of P-A axis responsiveness and avoidance outlined so far, shock has been the principal aversive stimulus. However, it has been found that a variety of stressors such as novel environment, cold swim, loud noise, bright light, and immobilization are effective in producing P-A responses (Manogue, Leshner, & Candland, 1975; Friedman & Ader, 1969; File & Peet, 1980; Lenox, Kant, Sessions, Pennington, Mougay & Meyerhof, 1980; Sithichoke, Malasanos & Marotta, 1978) implying a non-specific stress sensitivity of this system. The P-A axis has

been found responsive during avoidance situations where the subject is no longer receiving shocks (Friedman, Ader, Grota & Larson, 1967; Mason, Brady, & Tolliver, 1968). P-A responsiveness in the absence of physically noxious stimuli but in the presence of conditioned stimuli or novel circumstances has also been demonstrated. Friedman and Ader (1967) examined the rise in plasma corticosterone in animals exposed to shock and in animals merely placed in the shock chamber but not shocked and found comparable increases in plasma corticosterone not significantly different from each other but different from unmanipulated controls. File and Peet (1980) showed that exposure to an unfamiliar room produced increases in plasma corticosterone which were greater in bright than in dim light (light was used by these researchers as an independent variable for the manipulation of anxiety in their social model of anxiety).

DeWied (1977) expressed the belief that a plausible explanation for the "short term" effect of ACTH and related peptides seemed to be a temporary increase in motivation. He cited a study by Stratton and Kastin (1974) where ACTH facilitated acquisition of a shuttle-box avoidance performance by rats trained at low footshock levels suggesting the possibility of an additive relationship of fear produced by footshock and that induced by ACTH injection.

Induction of fear by ACTH administration was also the conclusion of Bohus, Gispen and deWield, (1973); "...the short term effect of ACTH 4-10 seems to be the temporary restoration of fear motivation in hypophysectomized rats. The progressive but not sudden decrease of avoidance performance after cessation of treatment might be a reflection of short term normalization of fear drive and consequent learning ability" (p. 142). Weiss, et al., (1970) commented similary: "...we would suggest that in a stimulus situation which elicits fear ... the hormonal influences (ACTH and corticosterone) result in an increase or decrease in an animal's general level of fearfulness. Thus hypophysectomized animals (are) less afraid than normal animals whereas adrenalectomized animals (are) more afraid" (p. 867).

In their experiment, Weiss, et al., (1970) found that hypophysectomized animals showed attenuated avoidance behavior in both active and passive avoidance tests which could be reversed by ACTH. Supranormal responding was apparent in adrenalectomized animals which reverted to normal upon corticosterone administration. An interesting aspect of the Weiss et al. study was that the largest behavioral differences due to P-A axis changes resulted from experimental conditions where fear cues were minimized during passive avoidance and extinction of active

avoidance responses. Because hormonal mediation was found ineffective when fear was strongest just prior to shock onset, these investigators concluded that the P-A axis is most effective during circumstances of mild and generalized fear. During times of more intense fear it is overcome by fast acting neural mechanisms. This is interesting in view of the fact that benzodiazepine sensitve animal models of anxiety show greatest reliability in conflict tests which may constitute another situation giving rise to mild, generalized fear, or perhaps more meaningfully, ambiguous behavioral demands in the absence of a clearly localizable, distinctly aversive stimulus to which behavior can be referenced. It may be, as Weiss suggested, that in situations of intense fear, behavior, learned or species typical behavior, is demanded by mechanisms which are sensitive to neither P-A axis hormonal influence or the benzodiazepine system.

In a social interaction test of anxiety, $ACTH_{4-10}$ was found to reduce social interaction between pairs of male rats below levels seen for untreated animals (File, 1979). As with untreated controls, interaction time decreased across cage familiar-low light, cage familiar-high light, cage unfamiliar-low light and cage unfamiliar-high light conditions, but all interaction times were below that of controls.

Locomotion remained at normal levels so sedative effects were unlikely. According to the criteria of the researcher, the decrease in social interaction correlated with increases of anxiety (cage unfamiliarity and illumination) indicated sensitivity to anxiety-evoking stimuli was present under the influence of ACTH. This is in contrast to a loss of such sensitivity with benzodiazepines or ethanol in this same test (File, Hyde, & Pool, 1976). The overall lowered level of interaction time was considered to reflect an increased anxiety level produced by $ACTH_{4-10}$ evident in all conditions. Similar effects were found when $ACTH_{4-10}$ was administered intracerebroventricularly prior to the social interaction test further supporting central mediation of the lowered levels of interaction (File & Clarke, 1980). Due to these findings, ACTH was posited as an anxiogenic agent.

Corticosterone in intact animals was examined in the social interaction test (File, Velucci, & Wedland, 1979) and its effect was found to be similar to that of the benzodiazepines, i.e., anxiolytic. Adrenalectomized animals were also examined and found to engage in very little social interaction (File, 1979). These levels were so low it was concluded that lack of further reduction with increases in anxiety-evoking cues was probably due to a floor effect and did not

necessarily indicate lack of sensitivity to these stimuli. Corticosterone administration increased social interactions and reinstated the test condition sensitive pattern.

Animals receiving corticosterone replacement behaved like sham operated controls and also had plasma corticosterone levels indistinguishable from controls. These authors suggested that since ACTH displayed anxiogenic action and corticosterone anxiolytic activity in the social interaction test, the behavioral pattern of adrenalectomized animals was probably due to the combined effects of adrenalectomy, i.e., high ACTH levels, and low glucocorticoid levels.

## Benzodiazepine Effects

It has been found that anxiolytic agents including the benzodiazepines are effective in antagonizing the elevation in plasma corticosteroids usually occuring in response to stress. Lahti and Barsuhn (1974) demonstrated that rats moved into a novel environment did not show the usual degree of elevation of corticosteroids if they had been pretreated one hour previously with diazepam. Pretreatment with a neuroleptic, an antidepressant, an alpha blocker, a beta blocker, an anticholinergic, or a narcotic analgesic were all found ineffective. The benzodiazepines tested showed rank order potency in blocking adrenal

response correlated with clinical potencies. Administration of ACTH to benzodiazepine-pretreated rats elicited normal elevations of corticosteroids indicating benzodiazepine blocked ACTH release but not adrenal responsiveness to circulating ACTH.

Benzodiazepines have the same effect when the stressor is inescapable cold swim (LeFur, Guilloux, Mitrani, Mizoule, & Uzan 1979). Phenobarbitol and meprobamate were also effective but tricyclic antidepressants, MAO inhibitors, neuroleptics, and amphetamines were not. In this situation, also, adrenal responsiveness was shown to be normal when ACTH was injected into benzodiazepine-treated animals. The cold swim period was set at 5 min in duration in order to induce a moderately stressful situation, which these researchers considered to be psychological in nature, and unconfounded with the physical stress of muscular fatigue. Four different benzodiazepines were tested and all caused dose-dependent inhibition of plasma corticosteroid elevations. Relative potencies were found to correlate highly with relative potency to displace $^{3}$H-diazepam in radioreceptor binding assays and also with reported clinical potencies.

Barlow, Knight, & Sullivan (1979) examined corticosterone responses, with and without chronic diazepam, to three different stress conditions: a 95 dB SPL noise for 1 hr, a 4-sec, 30V foot shock twice per

min for 5 hrs, and immobilization for 0.5 hr. These conditions were posited by these researchers as graded in stress intensity, noise being the least stressful and immobilization the most stressful. Plasma corticosterone elevations were seen to be lowest but still significantly different from unstressed animals in the noise group, greater in the shock group and greatest in the immobilization group. Since the same animals were used in all the stress procedures in an uncounterbalanced manner, it was not clear that increasing corticosterone elevations resulted completely from differences in stress severity and not also from repeated testing. In any case, diazepam reduced corticosterone elevations elicited by noise by 30% and was ineffective in the other conditions. This may be due to the possibility that responses to mild stress are differently mediated than are those elicited by severe stress as suspected by Weiss et al. (1970). But, it may also indicate an effect on the first of a series of stress producing manipulations lost on subsequent ones.

Immobilization stress was also examined by Keim and Sigg (1977). In contrast to the findings of Barlow et al., diazepam in this study was found to inhibit corticosterone elevations due to immobilization. The immobilization technique in this study involved containment in a cylinder of 7cm diameter.

In the Barlow, et al. study the paws were fastened to a board with adhesive plaster. It may be suspected that paw fastening and whole body constriction in a small space differ considerably in the degree of stress impossed on a rat which typically seeks small spaces when alarmed. A milder fear produced by the latter type of immobilization may account for the sensitivity to diazepam treatment not seen in the paw entrapment procedure.

The course of changes in the concentration of plasma corticosterone for five hours following exposure to an electric current of 1mA for 1 sec every min for 30 min in rats pretreated with chlordiazepoxide or diazepam one hr prior to shock and in untreated controls was examined by Krulik and Cerny (1971). They found that in untreated animals circulating corticosterone levels increased 207% immediately after exposure to shock. Chlordiazepoxide significantly reduced this increase to about 150%. This difference in responsiveness to stress was less evident at the first and second hr post stress when corticosterone levels in both groups dropped to prestressed control levels but was again seen at three hours post stress when the untreated group showed elevations about 120% of unstressed controls and no increase was seen in the chlordiazepoxide group. Both groups had levels of about 50% increase at five hours

## Introduction to Biochemical Experiments

In endocrine systems, hormones are secreted into the blood where they are carried to the target organ(s). The blood is then a candidate for examination for substances which may have physiological effects on organs and systems regulated or modulated by the endocrine system. It is well documented that various hormones have effects upon central neural circuits independent of the negative feedback regulatory loop which may be essential for homeostasis. For this reason rat blood was examined for the existence of a factor with the capacity to inhibit tritiated benzodiazepine $^{3}$H-flunitrazepam (3H-flu) binding to rat brain synaptosomal fractions. Preliminary work indicated an inhibitory capacity of the blood. This work was replicated with further characterization of the inhibitory activity.

Initially, dose dependent inhibition of $^{3}$H-flunitrazepam binding by rat serum was demonstrated. A Scatchard analysis to determine the nature of the displacement and high performance liquid chromatography for determination of the molecular weight of the active blood borne factor was also performed. Factor stability with respect to heat and acid was investigated, and high performance liquid chromato-

graphy of products of heat and acid treated serum was employed to investigate possible changes in the elution profile of remaining activity when compared with that of untreated serum.

In view of the strong implication of both the P-A axis and benzodiazepine system in stress and evidence of an interaction of the two systems, the role of the P-A axis in the presence of a blood borne inhibitory factor was investigated by examining the blood of animals two weeks after hypophysectomy or adrenalectomy. Blood from these two groups was compared with two groups of sham operated controls for the capacity to inhibit $^{3}$H-flunitrazepam from binding sites in lysed synaptosomal membrane fragments from the brain of intact rats.

It was also decided to investigate the possibility that stress induced changes in binding may be mediated by P-A axis activity at the receptor level. Three brain regions; frontal cortex, hippocampus, and cerebellum, from hypophysectomized and adrenalectomized animals were tested for the capacity to bind $^{3}$H-flunitrazepam as compared with unoperated controls.

Since benzodiazepine binding has been demonstrated in both the central nervous system and in peripheral systems, the ability of rat serum to

displace $^{3}$H-flunitrazepam in the central nervous system and in a peripheral renal tissue known to contain benzodiazepine receptors was compared. Central nervous system specificity of the blood inhibiting factor was examined by comparison of its effects in rat brain frontal cortex and rat kidney tissue. To further examine specificity of the benzodiazepine binding inhibiting factor the capacity to inhibit $^{3}$H-imiprimine binding to presynaptic serotonergic sites on platelets and $^{3}$H-quinuclidinyl benzilate binding to muscarinic receptors in rat brain was investigated.

## Index of Biochemical Experiments

## Methods

### Animals

Male albino rats, 230-300 g in weight, of the Wistar strain purchased from Royalhart Farms were housed 4-6 per cage with food and water available at all times. Lights were on a 12-hour on/12-hour off illumination schedule.

### Preparation of $P_2$ Synaptosomal Membranes

Rats were sacrificed by decapitation and frontal cortex was removed by an oblique razor cut (approximately $30^{\circ}$ from vertical) at the furthest anterior extent of the caudate nucleus. This tissue minus the olfactory bulbs and tubercles was homogenized gently in 10 volumes of ice cold 0.32M sucrose and centrifuged twice at 1000 G for 10 min. Pellets were discarded and supernatants were recentrifuged at 30,000 G for 20 min to produce a crude $P_2$-synaptosomal fraction. The $P_2$-fraction was resuspended in 10 volumes of 50mM Tris-HCl buffer (pH 7.4) and stored in 3-ml aliquots at $0^{\circ}C$.

### Serum Preparation

Trunk blood was collected on ice from animals sacrificed by decapitation and immediately centrifuged at 1000 g for 15 min. Serum was then

stored at 4°C until assayed within 48 hours or treated by heat or trichloracetic acid (TCA) prior to storage. For heat treatment serum was immersed for 10 min in a 100°C water bath. The resulting gel was chopped by hand with a small spatula and then centrifuged at 25,000 G for 30 min. The supernatant was then stored (4°C) and assayed within 48 hr. Trichloracetic acid treatment consisted of addition of enough ice cold TCA to the serum to a final concentration of 5% TCA. This solution was mixed by hand adjitation and incubated on ice for 10 min. The supernatant after centrifugation at 15,000 g for 15 min was then stored at 4°C for assay within 48 hr.

## $^{3}$H-Flunitrazepam Binding Assay

Total binding of 1 nM $^{3}$H-flunitrazeman ($^{3}$H-flu) to diluted $P_2$ suspension was determined during each assay by including tubes containing only $^{3}$H-flu and cortical tissue preparation in Tris-HCl or $NaPo_4$/NaCl buffer in a total volume of 1 or 2 ml. Other paired tubes containing in addition 1 uM clonazepam, a concentration potent enough to displace all $^{3}$H-Flu from BDZ specific binding sites, were employed to determine the portion of total binding which was nonspecific. Binding carried out on kidney tissue employed RO5-4864, a more potent inhibitor of $^{3}$H-flu binding to peripheral tissues, instead of clonazepam. Specific

binding was defined as the total binding minus non-specific binding occuring in the presence of clonazepam.

All assays were performed on ice using ice cold glass test tubes. Incubation period was 30 min at a temperature of $0^{o}C$. Rapid termination of the reaction was achieved by filtration under vacuum through Whatman GF/C filters. Following two 5-ml washes with iced buffer (50mM Tris-HCl, pH 7.4, or 50 mM sodium phosphate with 200 mM NaCl, pH 7.0) the filters were placed into scintillation vials and after addition of 10 ml of Beckman Ready-Solv HP to each vial radioactivity was determined by conventional liquid Scintillation counting in a Beckman Scintillation Counter.

Displacement binding by serum or treated serum was measured by its addition to paired assay tubes containing dilute $P_2$ (5-6 mg protein/ml) suspensions of cortical tissue and 1 nM $^{3}H$-flu. Nonspecific binding was subtracted from the total binding achieved for these tubes in order to determine the value for specific binding in the presence of serum or serum derived displacers. The degree of inhibition of $^{3}H$-flu binding by this displacer was defined as the ratio of the amount of specific binding in the presence of the displacer to the amount of specific binding in its absence.

## High Performance Liquid Chromatography

Fractionation of heat treated and TCA treated serum was carried out using a Waters Associates ALC/GPC model 244 liquid chromatographic system equipped with a U6K sample injector, model 6000A solvent delivery system and a model 441 fixed wavelength detector. Separations were performed on a I-125 protein column with a fractionation range of 1000-80,000 daltons equipped with an in-line precolumn filter (2uM frit element). Elution was achieved by the use of an isocratic buffer system (50 mM $Na_3Po4$, pH 7.0, containing 200 mM NaCl). Samples were directly injected in a volume of 100ul for serum and 500ul for either heat treated or TCA treated serum. Flow rates were 1.0 ml/min with resultant column pressure of 1000 psi at room temperature. Peptide/protein peaks were monitored at 214 nm.

## SEP-PAK

Separation by relative polarity was achieved through the use of disposable reverse phase $C_{18}$ cartridges (SEP-PAK cartridges, Waters Assoc., Inc.) This process has been shown to be valid for the separation of neuropeptides (Gay and Lahti, 1981). Application of 2 ml of sample was by disposable syringe after cartridge preparation with 2 ml methanol and two washes

with 5 ml deionized water. A volume of 0.8 $cm^2$ reverse phase packing attracts hydrophobic constituents of the sample permitting elution of a much greater proportion of polar moieties. Assay of the eluate for the capacity to displace $^3$H-Flunitrazepam from benzodiazepine binding sites in the brain was then carried out.

Kidney Tissue Preparation

Kidney tissue was prepared according to the method of Taniguchi, Wang, and Spector (1981) except Tris-HCl buffer (pH 7.4) was used. A single kidney from four animals was removed from rats sacrificed by decapitation. Kidneys were immediately frozen on dry ice. Kidney tissue was prepared by homogenization of the four kidneys in 30 volumes of 50 mM Tris-HCl buffer and centrifuged at 49,000 g for 10 min. The pellet was then resuspended in 30 volumes of buffer and recentrifuged at the same speed. The pellet resulting from the second centrifugation was resuspended in 20 volumes of buffer and frozen in 4 ml aliquots for later use.

Serum Inhibition of $^3$H-flu Binding in Kidney and Brain

For comparison of $^3$H-flu binding inhibition by rat serum in brain and kidney scatchard analysis of $^3$H-flu binding in both tissues was carried out in

in order to determine $K_D$ and B max values in both tissues. Serum was then examined with each of these tissues in displacement assays utilizing 0.25 $K_D$ values appropriate to each and identical concentrations of receptors as could be calculated from B max values identified for each tissue in the Scatchard analysis.

In 1 ml assays for brain tissue 0.25 nM $^3$H-flu was used and 0.42 pM receptor proteins/tube. In the 1 ml assay for kidney tissue 0.3 nM $^3$H-flu was used and 0.41 pM receptor proteins/tube. Four different volumes of rat serum were examined: 25, 50, 100 and 200 ml/tube.

Platelet Membrane Preparation

According to the method of Wennogle, Beer and Meyerson (1981), platelet concentrates obtained from the New York Blood Bank were diluted 1:20 with 0.05M Tris, 0.12M NaCl, 0.005M KCl buffer (pH 6.8) containing the following antiproteases:

0.025 u/ml Aprotinin

0.5 ug/ml Pepslatin

2 x $10^{-5}$M Bacitracin

3 mM EDTA

1 mM EDTA

Polypropylene laboratory ware was exclusively used. Platelets in buffer were centrifuged at 2500 g for 10 min before resuspension into 50 volumes of buffer. Sonication for a total of 45 sec was carried out in 3 15 sec periods at 1 min intervals during which material was kept on ice. This procedure ruptures cells.

Recentrifugation and resuspension into buffer 2x at 1800 g for 20 min took place prior to adjustment of pH to 7.4 and dilution to 3.0 mg protein/ml (determined by Bio-Rad Kit). This preparation was stored until used at $4^{o}C$.

## $^{3}H$-Imiprimine Binding Assay

Assessment of 25ul of rat serum inhibition of $^{3}H$-imiprimine binding to human platelet membranes was carried out according to procedures described for $^{3}H$-flu binding. Three nM $^{3}H$-imprimine and 10 uM desprimine as a determiner of non-specific binding was used in a total assay volume of 250 ul. Buffer used in this assay was as described in the human platelet membrane preparation section.

## Blood Preparation for Hypophysectomized and Adrenalectomized Rats

Hypophysectomized, adrenalectomized, sham operated hypophysectomized and sham operated adrena-

lectomized, male, Wistar rats were purchased from the Charles River Farms. Hypophysectomized animals were maintained on 5% glucose water and adrenalectomized animals were maintained on 1% saline water for two weeks prior to sacrifice. All animals were group housed, 6 to a cage, with food available ad libitum and a 12-hr on/12-hr off illumination schedule. Three weeks after surgery animals were decapitated and trunk blood was collected into iced test tubes in three pools for each of the four groups, two animals per pool. Serum was prepared as described above in the serum preparation section.

Preparation of Three Brain Regions From Hypophysectomized and Adrenalectomized Animals

Surgically hypophysectomized or adrenalectomized male Wistar rats as well and unoperated controls were purchased and maintained as described in the immediately previous section. Three weeks post surgery animals were sacrificed by decapitation. Brains were rapidly removed and dissected on dry ice into cerebellum, frontal cortex, and dorsal hippocampus before quick freezing and storage at $0^{o}C$.

Prior to assay brain regions were thawed and homogenized in 100 volumes of Tris-HCl buffer (pH 7.4). In a 2 ml assay 500 ul of this crude membrane preparation was used to measure basal $^{3}H$-flu binding.

The three brain regions from three animals in each of the three groups were individually examined in duplicate. Protein concentrations were assayed by Method of Bradford. Results were calculated as pmoles $^{3}$H-flunitrazepam bound/mg protein.

Drugs

Tritiated drugs were purchased from New England Nuclear, Boston, Mass. and clonazepam and RO54864 were provided by Hoffman LaRoche Inc., Nutley, New Jersey.

## Results

### Experiment 1A

The addition of rat serum to $^{3}$H-flunitrazepam binding assays inhibited binding to lysed rat brain synaptosomal membranes in a volume-dependent manner. The degree of inhibition of three concentrations of $^{3}$H-flunitrazepam by several different volumes of serum is exhibited in Figure 1. Increased inhibition with increases in the proportion of serum in the assay tube is clearly demonstrated. It can be seen that as little as 50ul or 5% serum/tube produced over 45% inhibition of the $K_D$ value of 1 nM $^{3}$H-flunitrazepam binding and as much as 80% inhibition occurs at 200 ul. This procedure was replicated with 50-300 ul serum/tube two times with nearly identical results. Displacement of 1 nM $^{3}$H-flunitrazepam with 300 ul

serum/tube has been replicated seven times, always producing 80-90% inhibition. Figure 1 shows that potency of inhibition was inversely related to concentration of $^{3}$H-flunitrazepam.

Experiment 2A

Scatchard analysis performed in the absence and presence of 50 and 100 ul of rat serum revealed dose dependent increases in $K_D$ but no change in $B_{max}$ for $^{3}$H-flunitrazepam binding. These data demonstrate that the active fraction is a competitive inhibitor of $^{3}$H-flunitrazepam binding. Data from the Scatchard analysis are plotted in Figure 2.

Experiment 3A

In order to determine the molecular weight of the active constituent of rat serum, high performance liquid chromatography was performed using protein I-125 molecular weight sieving columns. Each fraction was assayed and most inhibition of $^{3}$H-flunitrazepam was found to be due to the presence of a single peak. In Figure 3, it can be seen that maximal inhibition of over 40 units (1 unit = 10% inhibition/ml serum) coincides with a single peak emerging from the column at 8-10 ml elution volume. When compared to the elution volumes of a range of proteins of various molecular weights this active peak co-eluted with

globular proteins of approximately 16,000 daltons. A smaller peak of inhibitory activity of about 2-4 units is also evident at 12-14 ml elution volume. This co-elutes with standards of 2,000 daltons.

Experiment 4A

To determine the heat sensitivity of inhibitory activity serum was subjected to 100°C for 10 min. As can be seen in Table 1 this treatment degraded over 95% of the BIF activity in two separate preparations.

Heat treated serum was subjected to high performance liquid chromatography fractionation on a I-125 column. A maximal inhibition of 4 units (1 unit = 10% inhibition/ml serum before heat treatment) at 8-10 ml constitutes a 90% loss in inhibitory capacity when compared to that occuring for untreated serum (Figure 4). The smaller peak with inhibitory capacity appears unchanged.

Experiment 5A

To test for sensitivity to acid precipitation rat serum was treated with 5% trichloracetic acid resulting in 50% to over 90% loss of activity in three separate preparations (see Table 1). High performance liquid chromatography on an I-125 column of trichloracetic acid treated serum again yields a double peaked inhibitory profile. One peak of about 1.5 units at 7.5 ml elution volume (corresponding to the

elution volume of over 16,000 dalton globular proteins) and a second peak of maximal activity of 3.5 units emerging at 13-14 ml elution volume (corresponding to the elution volume of 2,000 daltons molecular weight proteins) (Figure 5).

Experiment 6A

In order to determine the relative polarity of the blood inhibiting factor non-polar fractions were removed from serum by SEPAK $C_{18}$ cartridges. Assay of the resulting fraction yielded 100% recovery of activity.

Experiment 7A

Inhibition of $^{3}H$-flunitrazepam binding from lysed synaptosomal membranes prepared from frontal cortex of intact rats by serum prepared from the blood of hypophysectomized, adrenalectomized and sham operated controls of the two types can be seen in Table 2. It is clear that no significant differences appear either between operated and appropriate sham operated groups or between the two types of surgically altered animals.

Experiment 8A

Table 3 displays basal $^{3}H$-flunitrazepam binding in pmoles/mg protein in three brain regions of rats

that have been hypophysectomized, adrenalectomized or left intact. It can be seen that although basal binding in the hippocampus and frontal cortex is about twice that seen in the cerebellum no differences occur for any of these regions according to surgically altered condition.

Experiment 9A

No inhibition of $^{3}$H-flu binding in kidney tissue was observed in the presence of 25 or 50 ul of rat serum (see Figure 6). At these volumes 45% and 60% inhibition respectively was achieved in brain. For 100 ul no inhibition was seen in kidney and 46% for 200 ul. In the brain 76% inhibition occured for 100 ul and 82% for 200 ul.

## Introduction to Behavioral Experiments

The existence of neural substrates for anxiety-moderating drugs such as the benzodiazepines suggests the presence of a natural anxiety regulating system involving receptors in the central nervous system as well as chemical activators of these receptors. Since several researchers have concluded that the hormones of the P-A axis have, as part of their activity, an anxiety or fear-mediating function, as part of this study activity of these hormones was examined in a behavioral test specifically sensitive to anxiety moderating agents.

A modified version of an anxiolytic-sensitive test was necessary in order to detect anxiogenic effects of treatments as well as anxiolytic effects. This modification was defined by the first experiment and incorporated into the test for use in subsequent experiments.

Adrenocorticotropic hormone (ACTH) has been posited as an anxiogenic agent with the capacity to enhance avoidance responding with respect to noxious stimuli of mild intensity. In a social model of anxiety (File & Velluci, 1978), $ACTH_{4-10}$ was examined and found to affect behavior in a manner opposite to

benzodiazepine which is also active in this test. Since benzodiazepine appears to moderate anxiety and $ACTH_{4-10}$ appears to enhance anxiety it is reasonable to investigate the possibility that ACTH may be active in a different but more established test which has been specifically developed for benzodiazepine sensitivity and found very reliable in predicting anxiolytic activity in humans. For this reason, in Experiment 2B, I investigated the capacity of $ACTH_{4-10}$ to alter punished responding in a thirsty rat conflict test. The centrally active, peripherally inactive, $ACTH_{4-10}$ fragment was used and the modified version of the test arrived at in Experiment 1B was employed for two reasons. First, a mild punisher was needed to conform with the findings of others that hormones of the P-A axis exert their influence only in such cases. Second, the modified version offers an intermediate level of suppression against which increases as well as decreases in suppression are detectable.

To further test a possible interaction between the benzodiazepine system and $ACTH_{4-10}$ the ability of $ACTH_{4-10}$ to antagonize the effect of benzodiazepine on punished behavior was examined in Experiment 3B. This was thought important because several substances have been isolated from biological tissues which are themselves behaviorally inactive but have the capacity to

attenuate the activity of benzodiazepine as a releaser of punished behavior (Crawley & Goodwin, 1981; Guidotti, 1982). In addition, in a social interaction animal model of anxiety, File and Vellucci (1978) found that $ACTH_{4-10}$ antagonized the increased social interaction effect of chronic chlordiazepoxide treatment. In Experiment 3B a similar procedure was undertaken with the modified thirsty rat conflict test. The effects of acute administration of $ACTH_{4-10}$ against a background of 5 days of chlordiazepoxide treatment was compared with the effects of vehicle administration to animals also chronically treated with chlordiazepoxide. Chronic chlordiazepoxide produces enhanced punished responding which would be attenuated by an antagonistic action of $ACTH_{4-10}$.

It has been suggested that release of corticosterone elicited by $ACTH_{4-10}$ may have an effect opposite that of $ACTH_{4-10}$ by being anxiolytic in nature. File et al. (1979) found corticosterone produced benzodiazepine-like effects in the social interaction test, but Lippa, Greenblatt, & Pelham, (1977) in a version of a thirsty rat conflict procedure, found that neither hypophysectomy nor adrenalectomy affected conflict responding and that they both enhanced benzodiazepine-induced release of punished responding. Since both these surgical treatments reduce drastically the levels of corticosterone it may be

suspected that an antagonistic relationship exists between corticosterone and benzodiazepine with corticosterone perhaps representing an anxiogenic influence. Toward resolution of these conflicting interpretations, in Experiment 4B the effect of corticosterone in the modified thirsty rat conflict test was examined. This test with the modification arrived at in Experiment 1B is now sensitive to anxiolytic and anxiogenic treatments and represents a vehicle for elucidating the nature of corticosterone.

A punishment-reducing effect of hypophysectomy was found in rats by Bohus et al. (1973), De Wied and Bohus (1966), De Wied (1971), and Weiss et al. (1977). This effect is inferred from a decrement in active avoidance behavior. A punishment-enhancing effect has been seen or inferred after adrenalectomy (Weiss et al., 1970; File et al., 1979). Neither of these phenomena were replicated by Lippa et al., (1977) in a conflict test. The effects of these surgical procedures were re-examined in a thirsty rat conflict test modified according to the findings in Experiment 1B. In Experiment 5B, hypophysectomized or adrenalectomized rats were examined and punished responding was compared with that of sham operated controls. Surgically altered animals and sham operated animals were also examined after acute

treatments with chlordiazepoxide in an attempt to replicate the potentiating effects of these surgical alterations on the punishment reducing effects of benzodiazepine found by Lippa et al. (1977).

## Index of Behavioral Experiments

To summarize, five separate experiments were carried out in a modified version of a thirsty rat conflict test.

Experiment 1B...Test modification. Chlordiazepoxide and amphetamine effects.

Experiment 2B...Dose-response relationship for $ACTH_{4-10}$.

Experiment 3B...Effects of acute $ACTH_{4-10}$ with chronic chlordiazepoxide.

Experiment 4B...Dose response relationship for corticosterone.

Experiment 5B...Effects of hypophysectomy and adrenalectomy.

Experiment 1B

The tests sensitive to anxiolytic drugs which have been used usually measure drug-induced release of punished responding. In these tests the degree of release from punishment-induced suppression produced by benzodiazepine or other anxiolytics is positively correlated with the magnitute of suppression produced by punishment. To test for anxiolytic effects, then, a major degree of suppression produced by high intensity stressors is desirable. However, studies involving the influences of the P-A axis on anxiety have indicated that influences are maximal during moderate anxiety resulting from exposure to stressors of moderate intensity. In order to study this influence in a behavioral test it is important to utilize a punisher of moderate intensity. In addition, if a drug or treatment which may have either an anxiogenic or an anxiolytic effect is of interest a high level of suppression would produce a floor effect against which detection of further suppression incident to anxiogenic treatments would be difficult. In this case too an intermediate degree of suppression produced by moderate punishment would be more useful and should reflect three characteristics:

1. Suppression should be demonstrated by punished response levels significantly different from unpunished response levels.

2. Punished responses should be released from suppression to a significant degree by known, reliable anxiolytic agents in order to demonstrate test sensitivity to anxiolytic treatments.

3. Further suppression should be achievable by anxiogenic treatments to demonstrate sensitivity to such treatments.

Calibration of the thirsty rat conflict procedure (TRC) (Vogel et al., 1971) was carried out in order to determine a useful current level of moderate intensity with the above characteristics in mind. Response levels, measured by the number of response-contingent mouth shocks taken by thirsty rats licking a water spout, were obtained for shock intensities of 100, 200, 300, 400, 500, and 600 uA as well as in the absence of shock. Each of these current levels was examined after vehicle and chlordiazepoxide administration.

In order to fully meet the criteria set above, the test was also examined for sensitivity to agents which enhance the effects of punishment as may be measured by decreases in punished response rates. While specific anxiogenic agents have not been described, amphetamine, which is a central nervous system stimulant and usually acts to moderate extreme

response levels on a rate dependent basis (increases low levels and decreases high levels of operant responding) will reduce low levels of responding induced by punishment (Cook & Davidson, 1973) This effect of amphetamine appears to result from non-stimulatory activity since it has a dose-effect relationship with increased avoidance of punishment within dose ranges showing no dose effect function relative to activity (Longoni, Mandelli & Pessotti, 1973). In addition, it was found that rats trained on a multiple schedule of FR 10 food and footshock VI 30 (food) will show decreased responding during the punishment component without effecting the unpunished component (Cook & Davidson, 1973). Also, the fact that amphetamine dose-dependently antagonizes chlordiazepoxide-induced release from suppression in the TRC test (Clody, D., personal communication) indicates the two drugs may act in part at a common locus. These findings indicate that amphetamine produces effects in punishment paradigms opposite to that of benzodiazepine. This will be tested at the optimal shock intensity.

In the course of generating a shock intensity-response curve an opportunity to examine the relationship between punishment-induced suppression and benzodiazepine release of punished responding was presented. The TRC test permitted investigation of this

relationship in naive animals engaging in an unconditioned response. This was thought important because prior studies involved conditioned, drug experienced animals and experience may have contributed to the correlations found.

## Experiment 1B

### Methods

Male, Wistar rats weighing 200-500 grams were housed six to a cage with food and water freely available on a 12-hour on, 12-hour off illumination schedule. Forty-eight hours prior to testing animals were housed two per cage with food present, but deprived of water. At least sixteen animals comprised each group. (Discrepancies between this number and the number indicated in the results are due to animals which were unsuccessful in initiating the test.)

### Apparatus

The test apparatus consisted of a clear plastic chamber, 38 x 38 cm, with an adjacent black Plexiglas chamber, 10 x 10.5 cm, containing a metal drinking tube protruding 2 cm at a height of 3 cm above a grid floor which extended throughout both chambers. The

animal standing on the grid floor and in contact with the drinking tube completed a circut producing 7/sec pulsed inputs to a computerized (Digital) control and data acquisition system. Each of these pulses was considered equivalent to a lick because it has been found that rat lick bursts occur at a rate of 7/sec in a relatively constant fashion (Collier and Bolles, 1968). Alternate connections of the spout-rat-grid circuit to a constant current Grason-Stadler shock source permitted delivery of unscrambled shock of adjustable intensity.

Procedure

All drugs and deionized water vehicle were administered intraperitoneally in a volume of 1ml/Kg body weight. Absorption time for chlordiazepoxide in doses of 8 mg/Kg was 30 min, absorption time for amphetamine, 1 mg/Kg, was also 30 min. These drugs were supplied by Lederle Labs, Pearl River, New York.

All animals were deprived of fluids 48 hours before testing. Prior to placement in the test chamber, animals were injected and replaced into their home cages for the drug absorption time. Each animal was then placed in the test chamber and permitted 20 licks at the drinking tube before the first mouth shock of 2 sec maximal duration was administered. Actual shock duration was determined by the subject's

ability to withdraw from the drinking tube. Subsequent to test initiation by the first shock, every 20th lick was followed by mouth shock during a 3 min test period. Licks during shock were not counted. Subjects that did not complete the first 20 licks within the 5 min necessary for initiation of the test were removed and not considered in the results. The number of shocks taken by each subject was recorded.

Results and Discussion

Data were analyzed utilizing a one tailed Mann-Whitney U test. Comparisons were made between vehicle and drug treated animals at each shock intensity, and vehicle treated animals at all shock intensities and vehicle treated animals receiving no shock. The latter measure is considered to be of punishment induced suppression, and the former measure reflects release from suppression due to drug treatment.

Means and standard errors are illustrated in Figure 7. It can be seen that significant suppression represented by reduced responding in vehicle injected shocked compared to vehicle injected unshocked animals is evident at all shock levels including the lowest of 100 A ($p<.01$).

Figure 7 also illustrates significant chlordiazepoxide effects ($p<.005$) at shock intensities of 200 uA and above while increased responding seen in the

chlordiazepoxide group is not significant in the no-shock condition.

Figure 8 illustrates the relationship between the degree of suppression produced by punishment and the extent of release from suppression due to chlordiazepoxide treatment. These are positively related in a nonlinear fashion by the calculations:

$$\% \text{ Suppression at given shock intensity} = \frac{\text{number of 20 lick bouts in zero shock condition - number of shocks delivered}}{\text{number of 20 lick bouts in zero shock condition}} \times 100$$

$$\% \text{ Enhancement at given shock intensity} = \frac{\text{number of shocks delivered in CDZ group - number of shocks delivered in vehicle group}}{\text{number of shocks delivered in vehicle group}} \times 100$$

A "good" intermediate level of responding against which either increases or decreases should be detectable appears to be attained at 200uA. This current produced a response level in the vehicle control group significantly different from the no-shock level ($p < .005$) and constitutes a level distinquishable from further suppression produced by higher shock intensities. In addition, Figure 9 shows that at 200 uA amphetamine produced a significantly greater degree of suppression than that seen after vehicle administration ($p < .05$).

Use of 200 uA current level in the thirsty rat conflict test appears to produce a level of responding which can be increased or decreased by treatments which alter behaviors in a manner generally agreed upon as representing useful animal models of anxiety. This current level was employed in further examination exploring the role of pituitary and adrenal hormes in mediating anxiety as defined by this test.

## Experiment 2B

### Methods

In this experiment drug treatment differs but animals, apparatus, and procedure were as described for Experiment 1B. Drug treatment consisted of four concentrations of $ACTH_{4-10}$ (Peninsular Labs; San Carlos, Calif.) administered intraperitoneally just prior to placement in the apparatus. An absorption time of 3-5 min was permitted. On a single day 50 ug/kg and 75 ug/kg as well as a deionized water control group were examined. On another single day 100 ug/kg and 200 ug/kg along with a deionized water control group were examined. Injection volumes for all groups was 1 ml/kg. A current level of 200 uA was employed as determined in Experiment 1B. A total of sixteen animals per group were injected and placed in the

apparatus. Discrepancies between this number and the numbers reported in the results section equals the number of nonresponders, i.e. animals which failed to initiate the test period by performing 20 licks.

Results

Means, standard errors, and the number of animals in each group are exhibited in Figure 10. The two control groups for the two test days were pooled since they were not significantly different. No differences were found between $ACTH_{4-10}$ and vehicle (Mann-Whitney U).

Experiment 3B

Methods

Animals, apparatus and procedure were the same as described for Experiment 1B except 8 mg/kg of body weight of chlordiazepoxide was administrered to half the animals once each day for five days prior to test day. The remaining animals received deionized water in the same volume of 1 ml/kg. On the test day animals were injected intraperitoneally just as on the previous five days with chlordiazepoxide or deionized water and returned to the home cage for an absorption period of 30 min. Animals from both groups then were administered intraperitoneally 100 ug/kg $ACTH_{4-10}$ or deionized water and placed into the test chamber after an additional absorption time of 3-5 min. Animals

chronically treated with chlordiazepoxide or deionized water and acutely treated with $ACTH_{4-10}$ were run all on a single day. Animals chronically treated with chlordiazepoxide and deionized water and acutely treated with deionized water were all run on a different single day.

## Results

Means, standard errors and the number of animals which successfully initiated the test are displayed in Figure 11. Examination of this figure shows that $ACTH_{4-10}$ increased the number of shocks taken by animals chronically treated with chlordiazepoxide and those chronically treated with water. However, this difference did not reach statistical significance for the chlordiazepoxide group ($.05<p<.1$) when analyzed by a two-tailed Mann-Whitney U test. The difference between acute $ACTH_{4-10}$ and vehicle in the chronic vehicle treated group was statistically significant ($p<.02$).

## Experiment 4B

Except for drug treatment animals, apparatus and procedure were the same as described in Experiment 1B. Prior to placement in the test chamber 2.5, 0.5 or 0.10 mg/kg of corticosterone was administered intraperitoneally in a volume of 1 ml/kg and permitted an

absorption period of 20 min. One group was given deionized water vehicle in the same volume. Animals were replaced in the home cage after drug administration for the absorption period. One water injected control group and the dose of 2.5 mg/kg were run on a single day and the remaining drug groups and a second control group were run on a second single day.

## Results

The data are displayed in Figure 12. The Mann-Whitney U test indicated that performances did not differ significantly among the groups.

## Experiment 5B

## Methods

For this experiment 36 surgically hypophysectomized and 36 surgically adrenalectomized male Wistar rats were obtained from Charles River Farms. These animals were 7 weeks of age at time of surgery and 21.5 weeks of age at time of testing. Animals were housed as described in the previous experiments except that 1% saline water was provided for the adrenalectomized animals and 5% sucrose water was provided for hypophysectiomized animals in place of the normally provided tap water. Sham operated control animals of both types (total N=72) were also

purchased and delivered at the same time and maintained in the same manner but with tap water provided. Food for all animals was available at all times.

Animals were water deprived and tested as described above for other experiments. Each group received either chlordiazepoxide (8 mg/kg, in a volume of 1 ml/kg) or a control injection of deionized water administered intraperitoneally 30 min prior to testing. All animals were replaced into their home cage for the absorption period from which they were removed for placement in the test chamber. All eight groups were run on the same day. Test apparatus was the same as that described for Experiment 1B.

Results

Ten hypophsectomized animals survived the water drprivation period. Surviving animals appeared lethargic and showed retarded righting responses which were exacerbated by chlordiazepoxide treatment. The twenty surviving adrenalectomized animals appeared normal before and after chlordiazepoxide treatment. Because of the limited number of animals surviving water deprivation eight, hypophysectomized animals and ten adrenalectomized animals for each group were tested.

Data from Experiment 5B are shown in Figure 13 and Figure 14. Adrenalectomized animals (Figure 13) showed a significant benzodiazepine effect ($p<.05$, Mann-Whitney $U$) as did the sham operated controls ($p$ .05). Comparisons between responding in the two chlordiazepoxide groups and between the two control groups treated with deionized water reveal no differences of significance. Two sham operated animals treated with chlordiazepoxide failed to respond.

Three chlordiazepoxide treated hypophsectomized animals successfully initiated the test and seven vehicle treated animals were successful. As can be seen in Figure 14 hypophysectomized animals exhibited no benzodiazepine-induced release of punished responding. In the sham operated hypophysectomized groups, two did not respond in the chlordiazepoxide group in addition to one non responder in the deionized water greated group. A significant ($p$ .05) benzodiazepine effect is evident when chlordiazepoxide treatment is compared with vehicle treated in the sham hypophysectomized animals.

## DISCUSSION

Several studies carried out by several researchers have suggested an important interaction between the benzodiazepines and the P-A axis. Two of several possible loci for this interaction involve alterations in benzodiazepine receptors and/or in levels of endogenous ligands for these receptors. In the present study I have documented the ability of crude rat serum to inhibit benzodiazepine binding. This inhibition was much greater in brain than in kidney. Since receptors in the brain are pharmacologically and biochemically different from those in kidney, these results would suggest selectivity for brain benzodiazepine receptors. Scatchard analysis of $^{3}$H-flu binding in the absence and presence of rat serum indicates an increase in $K_d$ with no change in $B_{max}$. This competitive type of interaction suggests the presence of common binding sites.

Chromatographic analysis of crude rat serum demonstrated the presence of two peaks of inhibitory activity; one approximately 16000 daltons and the other approximately 2000 daltons. Biochemical characterization of crude rat serum revealed a loss of activity after heat or acid treatment. This change was due to the degradation of the larger (16,000 dalton) peak of activity. Preliminary results also

indicate that the larger fraction is degradable by papain suggesting a proteinaceous composition. These results imply the presence of a hormone-like substance in rat blood which may act as an endogenous ligand for benzodiazepine receptors.

In order to further examine the specificity of rat serum for benzodiazepine receptors the binding of $^{3}$H-QNB to whole brain and $^{3}$H-imiprimine to human platelet membranes was also examined. Unexpectedly, crude rat serum inhibited $^{3}$H-QNB and $^{3}$H-imiprimine binding to the same extent as $^{3}$H-flu binding. While these results would tend to diminish the concept of a selective action on benzodiazepine receptors several points should be made. First, not all binding systems were equally affected by rat serum. While $^{3}$H-flu binding to brain was inhibited by serum to an extent comparable with $^{3}$H-QNB and $^{3}$H-imiprimine, $^{3}$H-flu binding to kidney was not. These results exclude non-specific displacing activity. Second, serum is a highly heterogeneous tissue containing various substances which might affect different binding sites through different molecular entities. Third, preliminary data indicate that human plasma inhibits $^{3}$H-flu binding but not $^{3}$H-imiprimine binding. Clearly, in order for future work to be meaningful analysis of purified fractions is necessary. Purification of the two peaks is the next step for this

characterization and also for pharmacological testing.

Comparing the so far known characteristics of the blood borne factor with the characteristics of inhibiting factors found by others shows that identity of the smaller peak as an acid stable purine like inosine or hypoxanthine with molecular weights of 500 daltons cannot be ruled out. Woolf and Nixon (1981) described their 'nepenthin' as having a molecular weight around 16000 daltons, but it was heat stable and ours is not. Extractions from cerebrospinal fluid show displacement activity in fractions containing 700-3600 dalton substances and also entities above 10,000 daltons.

Two possible loci of interaction between the P-A axis and the benzodiazepine system are the benzodiazepine receptor and levels of circulating endogenous ligand(s). Each of these was examined in animals with and without pituitary or adrenal glands. Neither hypophysectomy nor adrenalectomy affected either of these parameters, diminishing their candidacy as sites of interaction.

In order to study the question of benzodiazepine systems and P-A axis interaction on a behavioral level an animal model of anxiety was used. The first experiment was designed to examine the relationship between response suppression by punishment and benzo-

diazepine-induced release of punished responding. A correlation between suppression and enhancement has been seen by other researchers in operant experimental procedures where well trained animals were offered high reinforcement rates accompanied by response contingent punishment in a multiple schedule along with a period of lower available reinforcement density in the absence of a punishment contingency. In these studies involving conditioned behavior, trained animals are used repeatedly and despite 'wash-out' periods intervening between drug treatments a question remains concerning the occurrence of effects resulting from multiple dosing in the same animals. These effects would not be controlled by counterbalancing. Benzodiazepine effects are known to differ according to prior drug experience. Both the social model of anxiety and the thirsty rate conflict model (TRC) employ an unconditioned response and drug-naive animals. The present finding of a strong relationship between punishment-induced suppression and benzodiazepine release of punished responding further validates benzodiazepine specificity for punished behavior. This finding also characterizes benzodiazepine sensitivity in this test as similar in nature to that seen in the other test types.

No major effects on punished responding were found for ACTH or corticosterone. A possible

anxiolytic effect of ACTH administration to animals with prior experience of five days of daily administration of either chlordiazepoxide or deionized water is contrary to expectations derived from the work of other investigators. This finding must be considered tentative until a replication has been carried out. However, a sensitizing effect for stressful manipulations is not unprecedented. Antelman, Eichler, Black and Kocan(1979) described a similarity in neurochemical and behavioral effects of at least some stressors and of stimulants like amphetamine. (Recall that amphetamine suppressed responding in TRC.) Amphetamine and some stressors were interchangeable in their ability to produce an augmented response to subsequent stress or amphetamine administration (sensitization). In the case of Experiment 3B it appears that some enhancement of suppression in chronically dosed animals may have occurred. Figure 15 shows mean numbers of shocks taken by animals in all of the control groups acutely treated with deionized water during the other experiments. Since all experiments were run on different days this represents an examination of day effect. Figure 15 also shows the mean number of shocks taken by the 15 animals chronically and acutely treated with deionized water. As can be seen, there is some evidence for enhanced suppression. Even though chronically treated animals did not show

suppression statistically different from all other groups, they did from some.

The role of ACTH as an anxiolytic in the sensitized animal and not otherwise must be replicated before being seriously considered. One possible mechanism for this effect may relate to the observation that high levels of ACTH produced by exogenous adminstration or endogenously by adrenalectomy (or possibly by five days of drug injection) uncovers a population of high capacity, low affinity $^{3}$H-GABA binding sites not detectable in untreated animals (Kendall, McEwen, & Enna, in press). In view of an intimate but not yet fully characterized relationship between the benzodiazepine and GABA receptors this population of new GABA binding sites may represent an important link between GABA and benzodiazepine/pituitary-adrenal interactions.

P-A axis hormones were not found active in the TRC test as they were in File's social model of anxiety. The presence of a group of differing animal behavior test designs all specifically and similarly sensitive to several agents having a particular psychoactive effect in humans suggests the existence of a mechanism manipulated by these tests and these agents. In this case, animal tests embody anxiety eliciting situations and the active agents are anxiety

reducing in humans. Hence, these tests are conceptualized as animal models of anxiety. The presence of agents which are active in one or some models of anxiety, but not in all well-established, reliable models of anxiety offers an opportunity for investigating the mechanisms underlying the behaviors elicited by the various test situations, and it may also call for a redefinition of the phenomenon considered to be commonly modeled by these tests i.e., anxiety. Common specific benzodiazepine sensitivity in tests of differing designs has led to a concept of anxiety as an intervening variable. To the extent that treatments are effective in all the tests in producing dependent variable alterations the concept of a unitary intervening variable is useful. Treatments which are effective in one test and not in another question a unitary intervening variable. Activity of ACTH in one benzodiazepine-sensitive test and not in another opens the question of whether these conflict tests represent models of anxiety in the sense of a single unitary variable or would be more usefully conceptualized as pharmacological models. The ability of an agent to prevent convulsions induced by pentylenetetrazol injections is also highly predictive of anxiolytic activity, reflecting a pharmacological model for the prediction of anxiolytic activity. A

newly synthesised triazolopyridizine is punishment reducing in behavioral tests but does not produce the sedative and ataxic side effects incident to benzodiazepine administration (Lippa et al., 1979). This drug is thought to discriminate between the anxiety mediating aspects of the benzodiazepines and the other effects by acting at the neural substrate of the former and not of the latter. Interestingly, it is also active as an antagonist of pentylenetetrazole-induced convulsions (Lippa et al., 1979).

If we were to fractionate benzodiazepine effects into an anxiety-mediating component and a component producing the side effects of ataxia and sedation, pharmacological evidence indicates the anti-convulsant effect would have to fall into the former although it appears far removed from a concept of anxiety as embodied in approach-avoidance animal tests. Fractionating the benzodiazepine effects in this manner is not only indicated by activity of triazolopyridizines but also by very strong evidence for multiple benzodiazepine receptors differentially involved with at least these two components of benzodiazepine activity (Klepner et al., 1979; Lippa et al., 1979; Lippa, Meyerson & Beer (in press).

Evidently some common site of action of anti-convulsant and anxiolytic agents permits use of the

pentlyenetetrazole tests as a predictor. Behavioral tests, although perhaps less arbitrary than this, are probably overestimated as anxiety models and underestimated as pharmacological models. Further counter-indications for defining the behavioral tests as anxiety models are seen in experimentation which demonstrates that stressor characteristics such as shock, conspecific interactions, or manipulations of ambient environment differentially affect physiological responses including those of the adrenal gland (Riley, Fitzmaurice, & Spackman 1981). Since benzodiazepine-sensitive behavioral tests variously utilize these variables, it seems unlikely that a simple unitary psychophysiological state is induced by these varied situations. The lack of consistent P-A axis hormone effects across benzodiazepine-sensitive tests probably indicates that these tests are not effective in imposing upon the animal a common state characteristic. Wider exploration of the parameters of each test is an essential consideration.

Table 1

Effect of heat (100°C/10 min) and trichloracetic acid (5%) treatment on inhibitory activity of rat serum (1 unit = 10% inhibition/ml sample).

| Units in Untreated Serum | Treatment | Units Remaining | Recovery of Activity |
|---|---|---|---|
| 789.00 | Heat | 23.10 | 2.91 |
| 819.00 | Heat | 35.10 | 4.28 |
| 394.00 | TCA | 93.80 | 23.77 |
| 518.70 | TCA | 38.00 | 7.32 |
| 750.00 | TCA | 91.20 | 12.16 |

Table 2

Inhibitory units in serum prepared from blood of three animals each of hypophysectomized, adrenalectomized or sham operated control rats, (1 unit = 10% inhibition/ml serum).

| | Altered | Sham Operated |
|---|---|---|
| Hypophysectomized | 30.6 | 28.7 |
| | 29.3 | 28.3 |
| | 29.3 | 29.6 |
| Adrenalectomized | 27.0 | 28.0 |
| | 28.0 | 28.0 |
| | 28.3 | 28.0 |

Table 3

Basal $^{3}H$-flunitrazepam binding to three brain regions of hypophysectomized, adrenalectomized or unoperated control rats in pmoles/mg protein.

Three brain regions in each of 3 an mals per group were examined. Binding in each region for each animal is separately displayed.

| | CEREBELLUM | HIPPOCAMPUS | CORTEX |
|---|---|---|---|
| CONTROL | 0.13 | 0.27 | 0.29 |
| | 0.22 | 0.28 | 0.32 |
| | 0.14 | 0.31 | 0.32 |
| HYPOX | 0.16 | 0.32 | 0.30 |
| | 0.15 | 0.46 | 0.30 |
| | 0.16 | 0.28 | 0.32 |
| ADX | 0.16 | 0.29 | 0.32 |
| | 0.10 | 0.32 | 0.31 |
| | 0.16 | 0.34 | 0.35 |

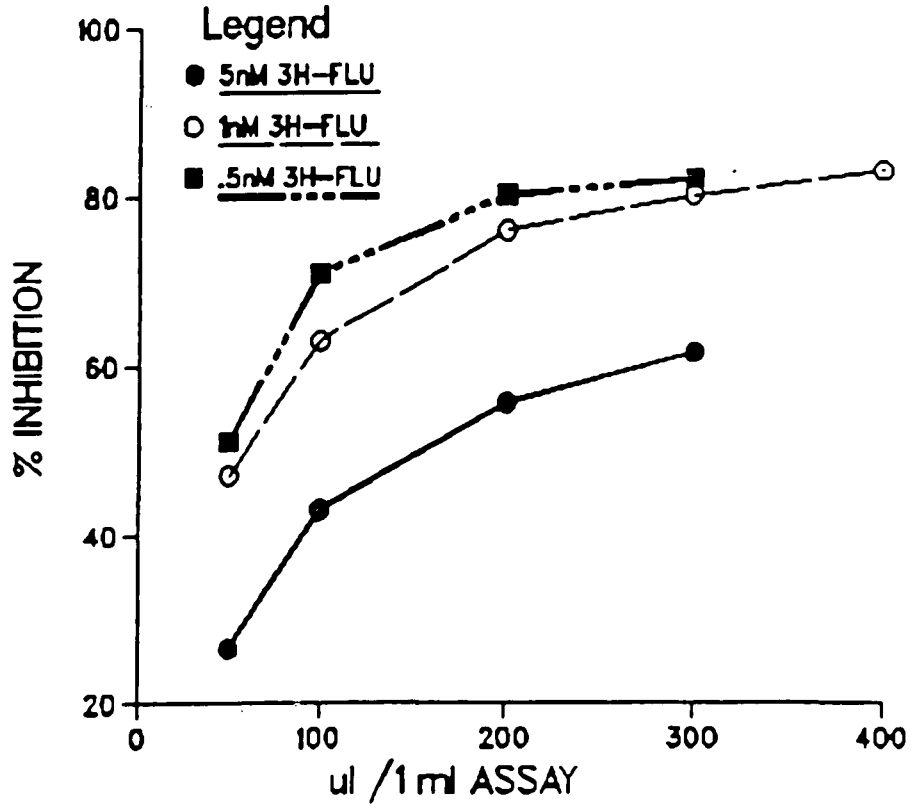



Figure 1

Displacement of three concentrations of tritiated flunitrazepam with four volumes of rat serum.

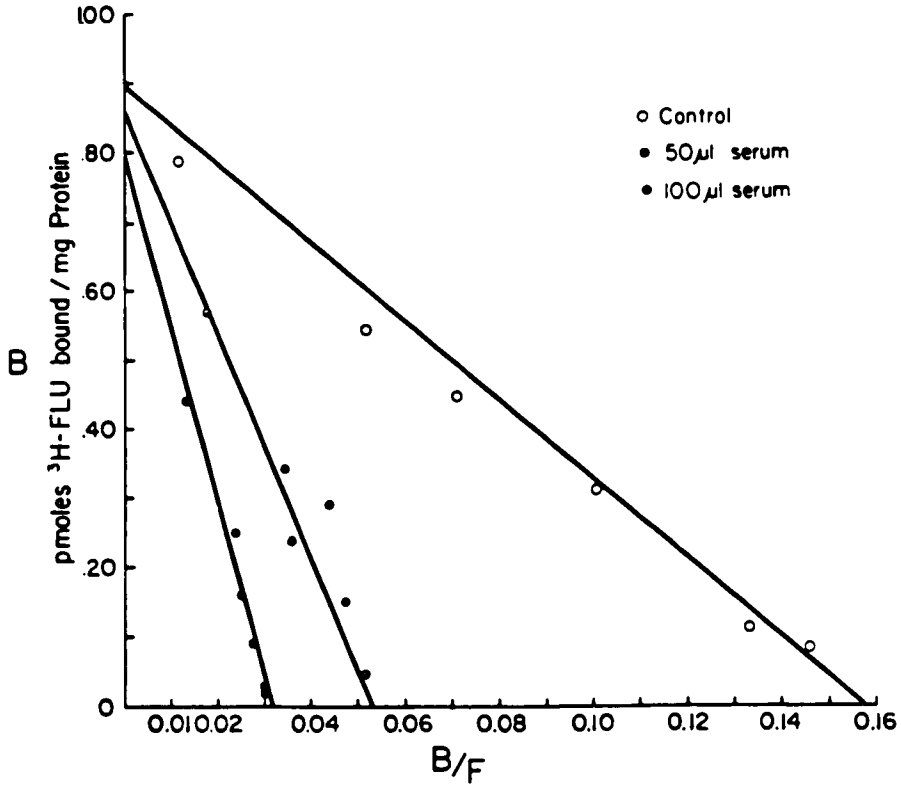
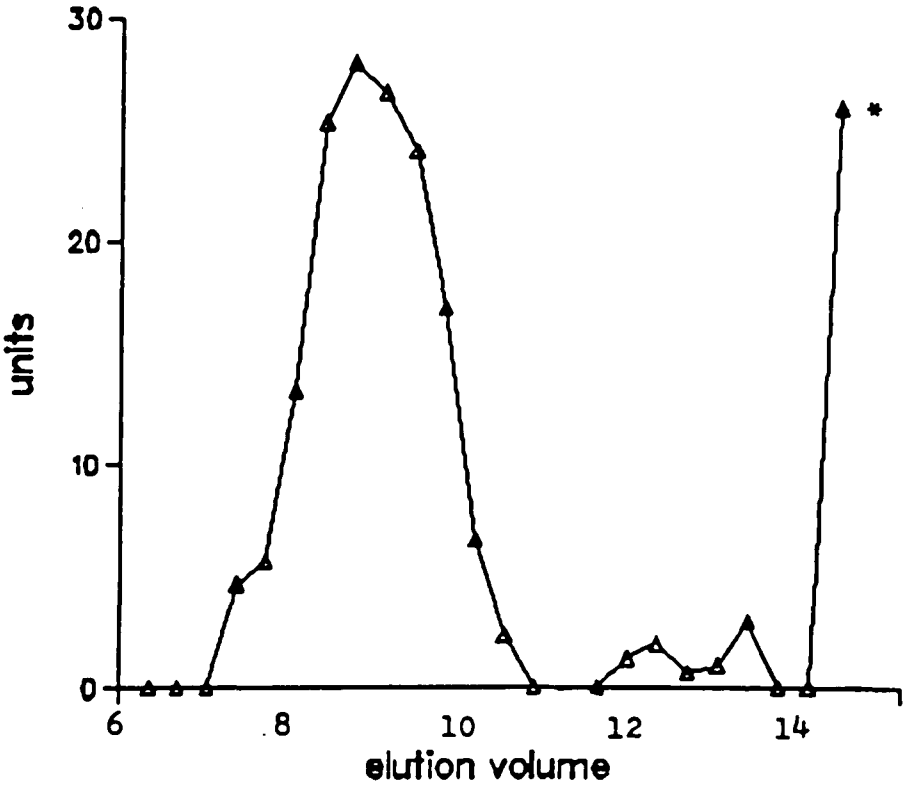



Figure 2

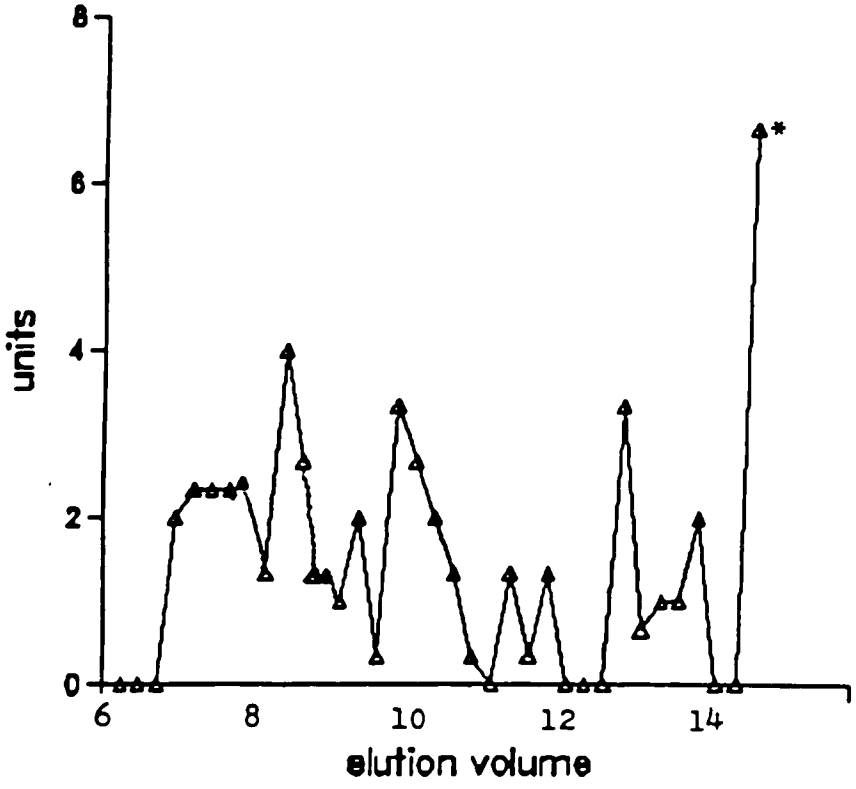
Effects of rat serum on binding parameters for tritiated flunitrazepam as dtermined by scatchard analysis.




*Inhibition by sample before fractionation

Figure 3

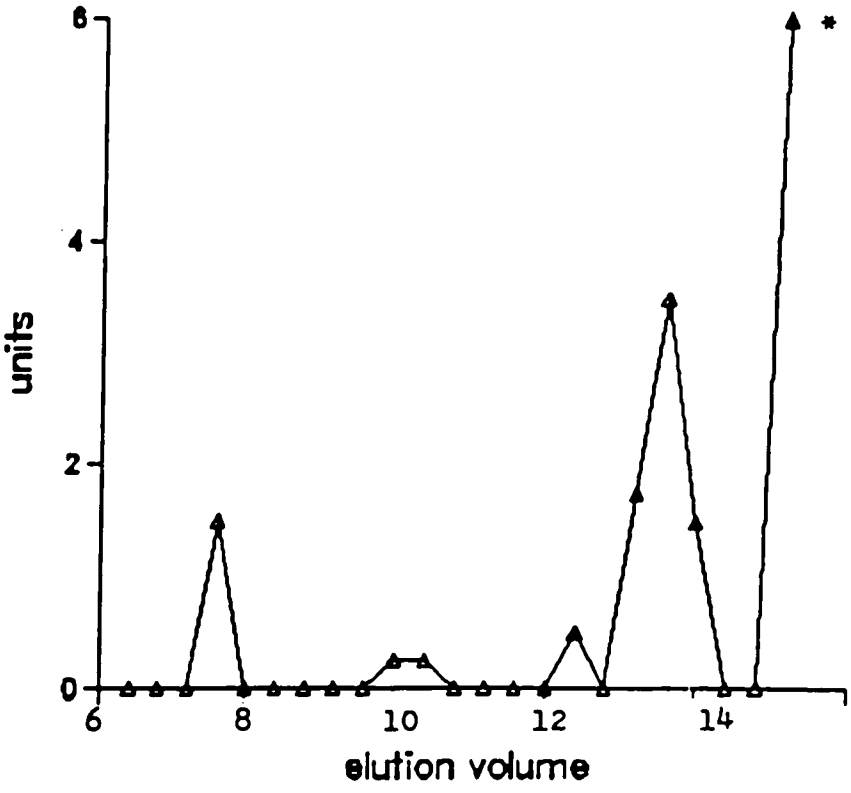
Activity of blood inhibiting factor in HPLC (I-125) fractions of rat serum ( 1 unit = 10% inhibition/ml).




*Inhibition by sample before fractionation

Figure 4

Activity of blood inhibiting factor in HPLC fractions of heat treated rat serum.




*Inhibition by sample before fractionation

Figure 5

Activity of blood inhibiting factor in HPLC fractions of trichloracetic acid treated rat serum.

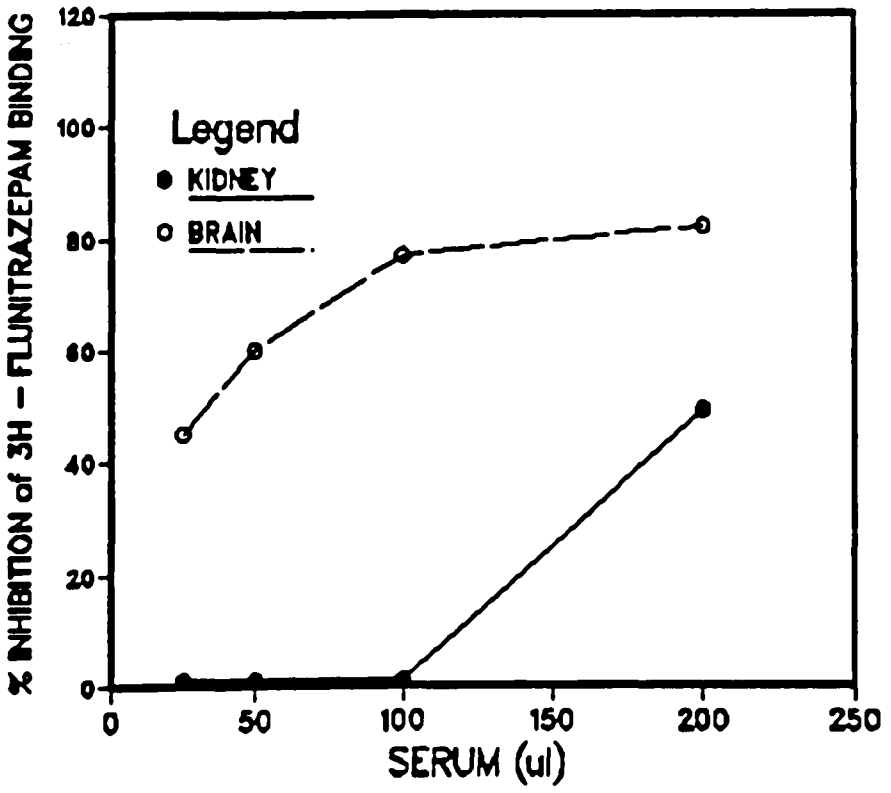
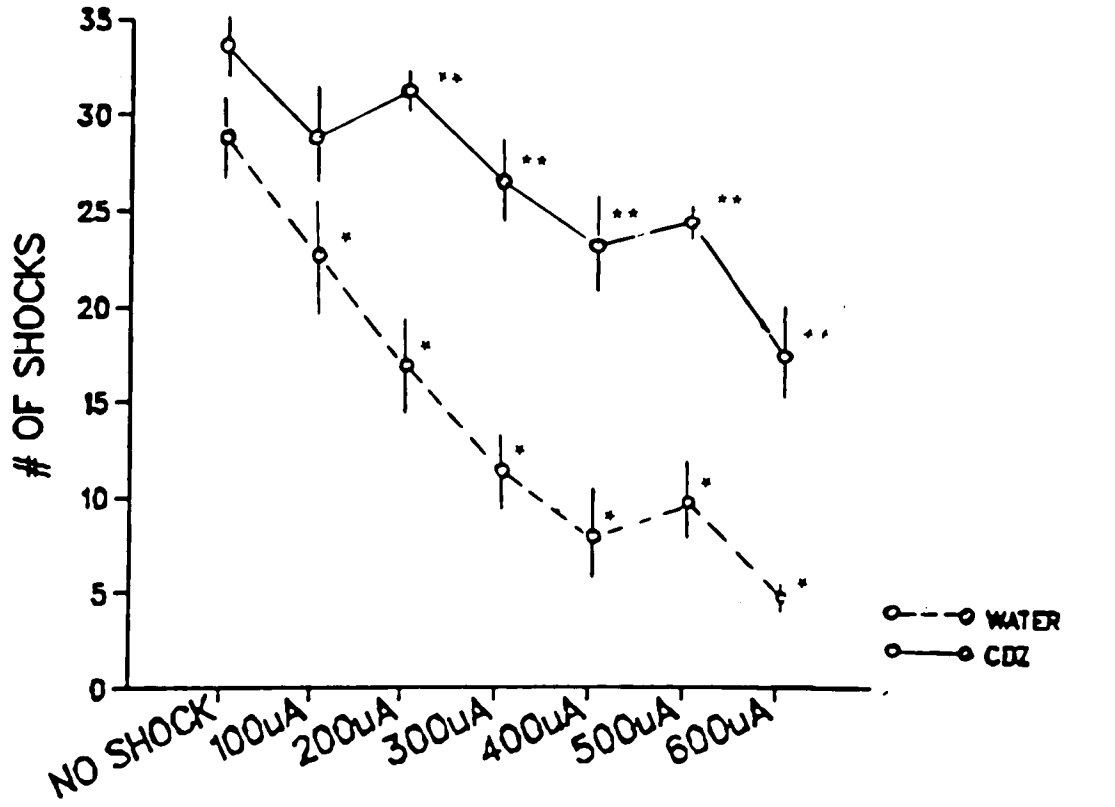



Figure 6

Inhibition of $^{3}$H-flunitrazepan binding in brain and kidney tissue by four volumes of rat serum.




* p<.01 (shock-induced suppression with respect to responding in the absence of shock)

** p<.005(release from suppression by chlordiazepoxide)

Figure 7

Current-response relationship in the thirsty rat conflict test (TRC). Means and standard error (s.e.).

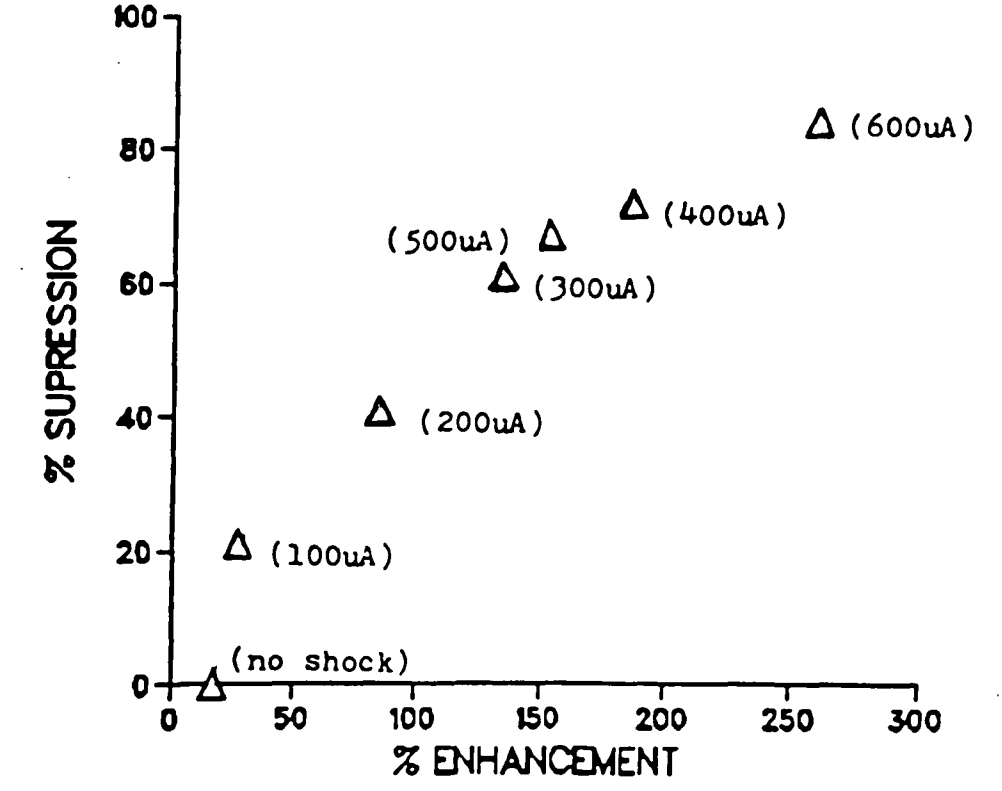
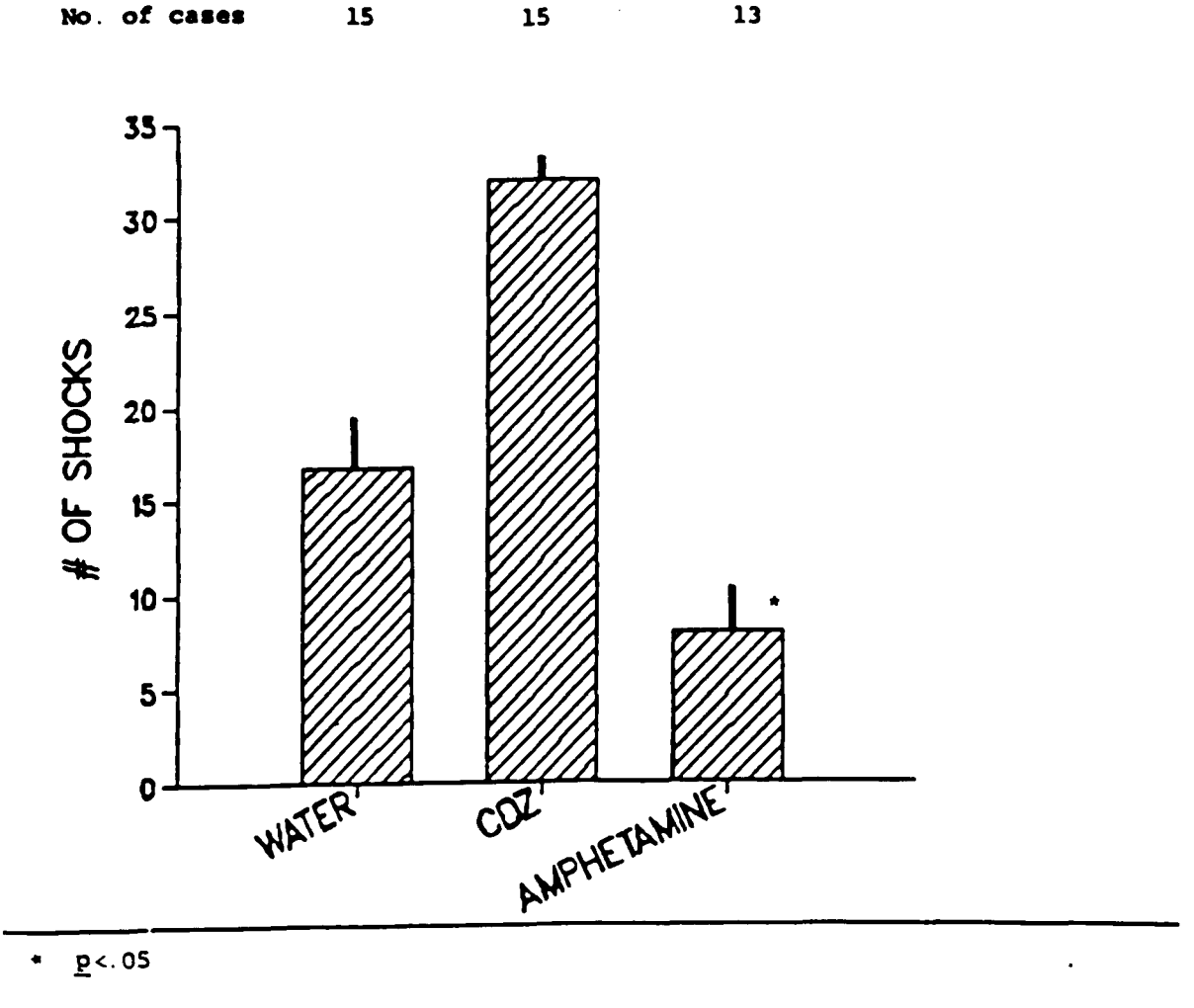



Figure 8

Relationship between mean degree of suppression and of enhancement induced by chlordiazepoxide administration on responding in TRC.




* $p<.05$

Figure 9

Comparison of effects of amphetamine treatment with chlordiazepoxide treatment in TRC (Means + s.e.).

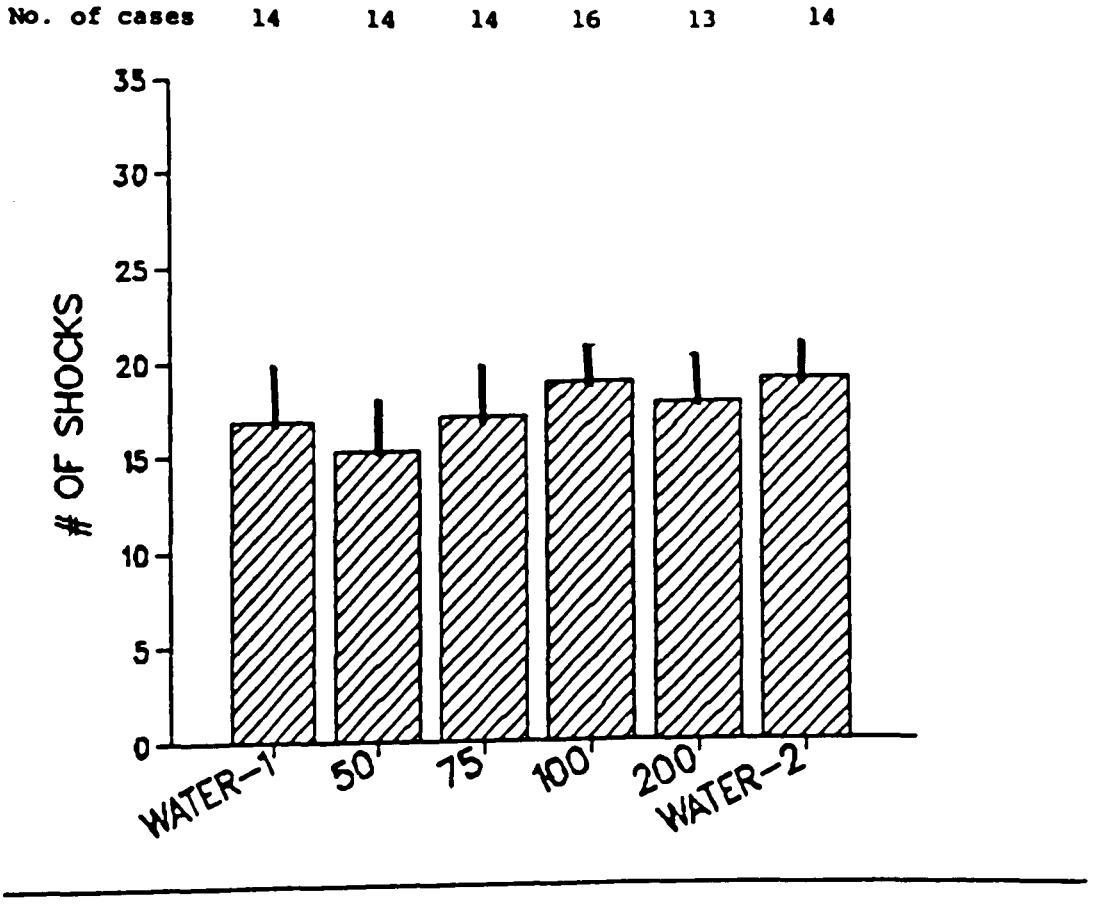
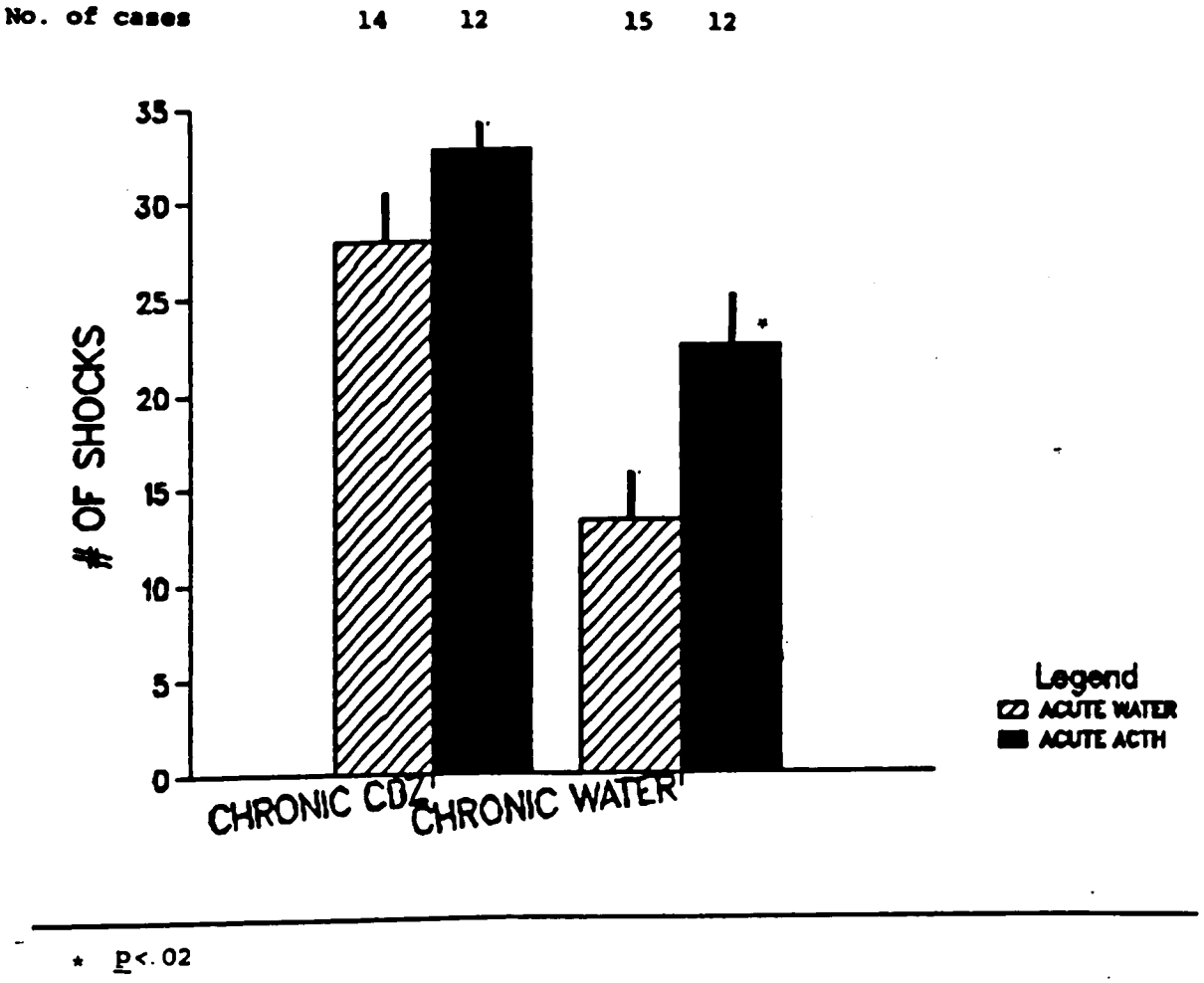



Figure 10

Dose-response relationship of four doses of $ACTH_{4-10}$ in TRC (Means + s.e.).




* $p < .02$

Figure 11

Effect of acute $ACTH_{4-10}$ against a background of chronic chlordiazepoxide administration on responding in TRC. (Means + s.e.).

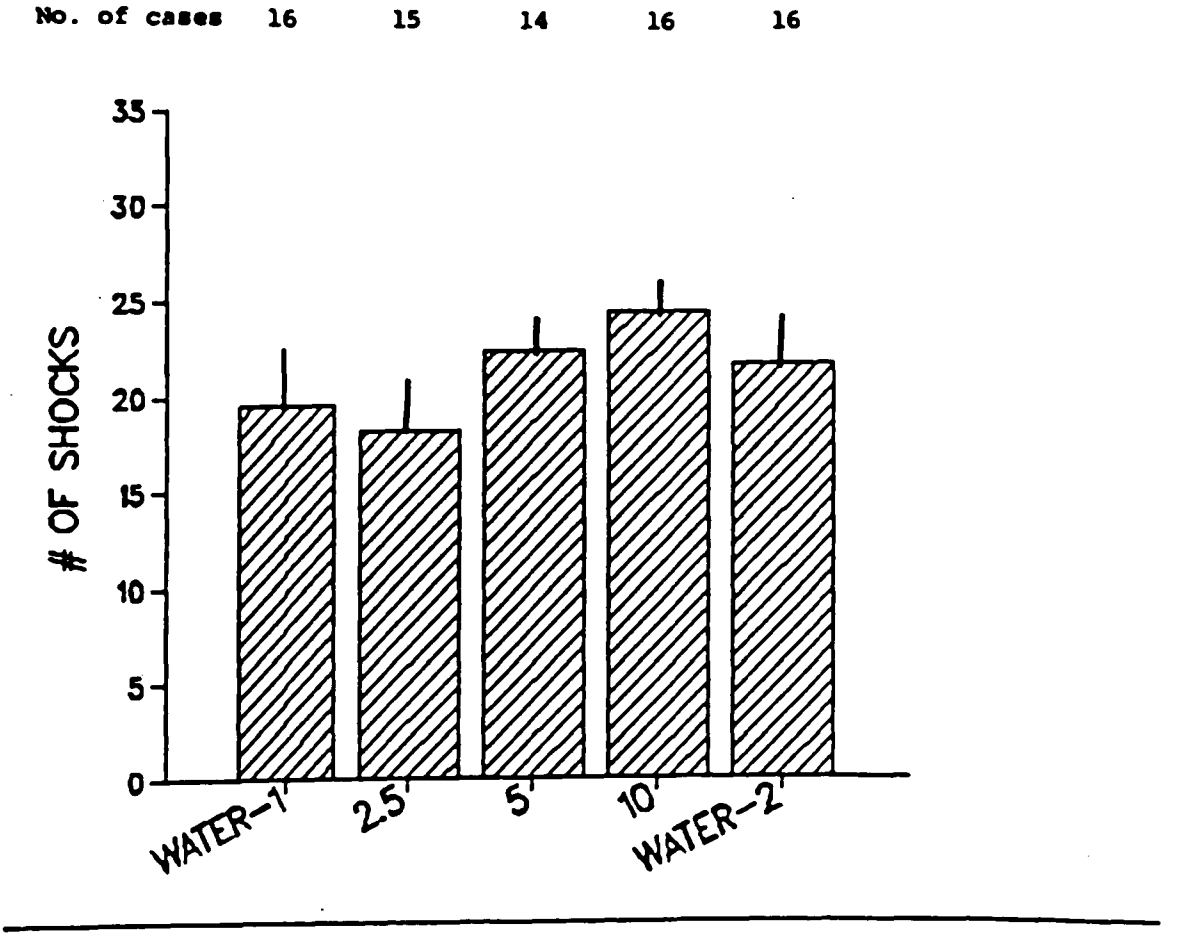
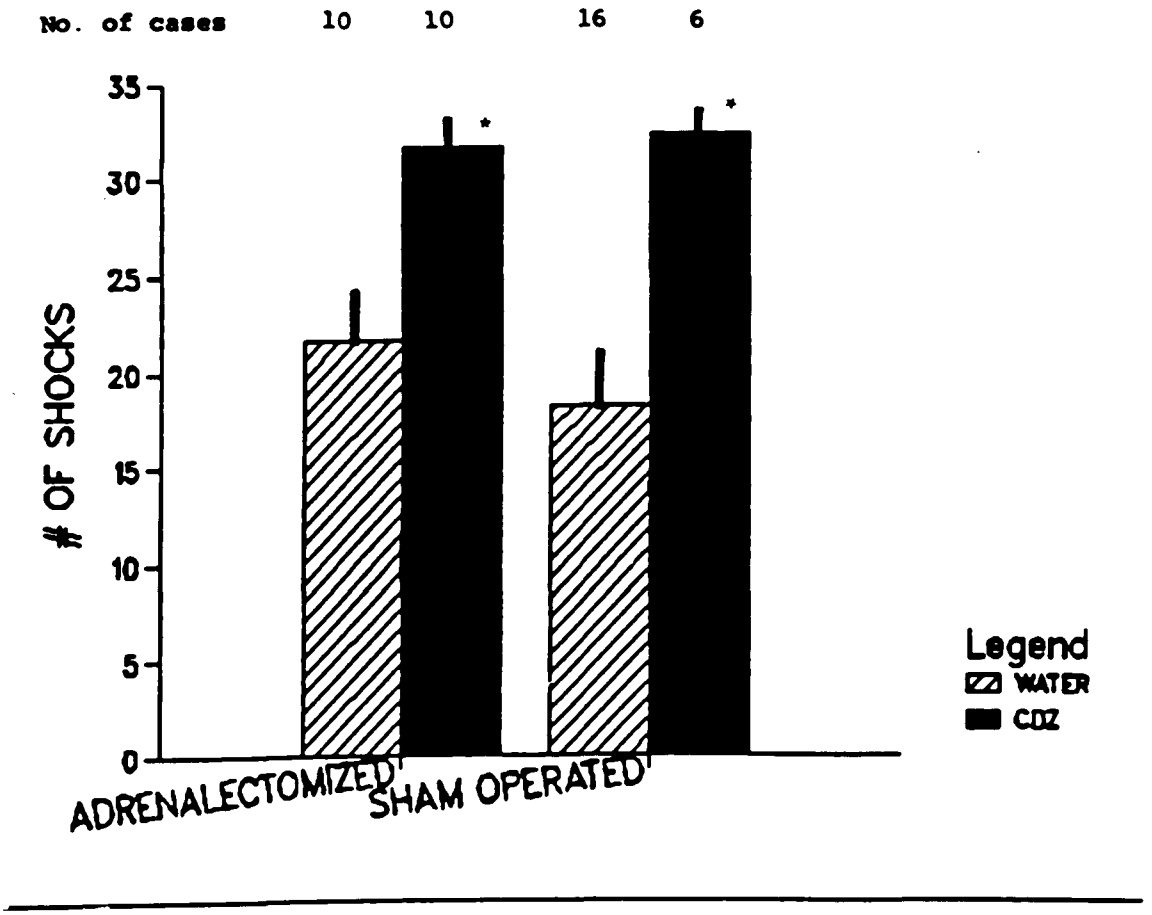



Figure 12

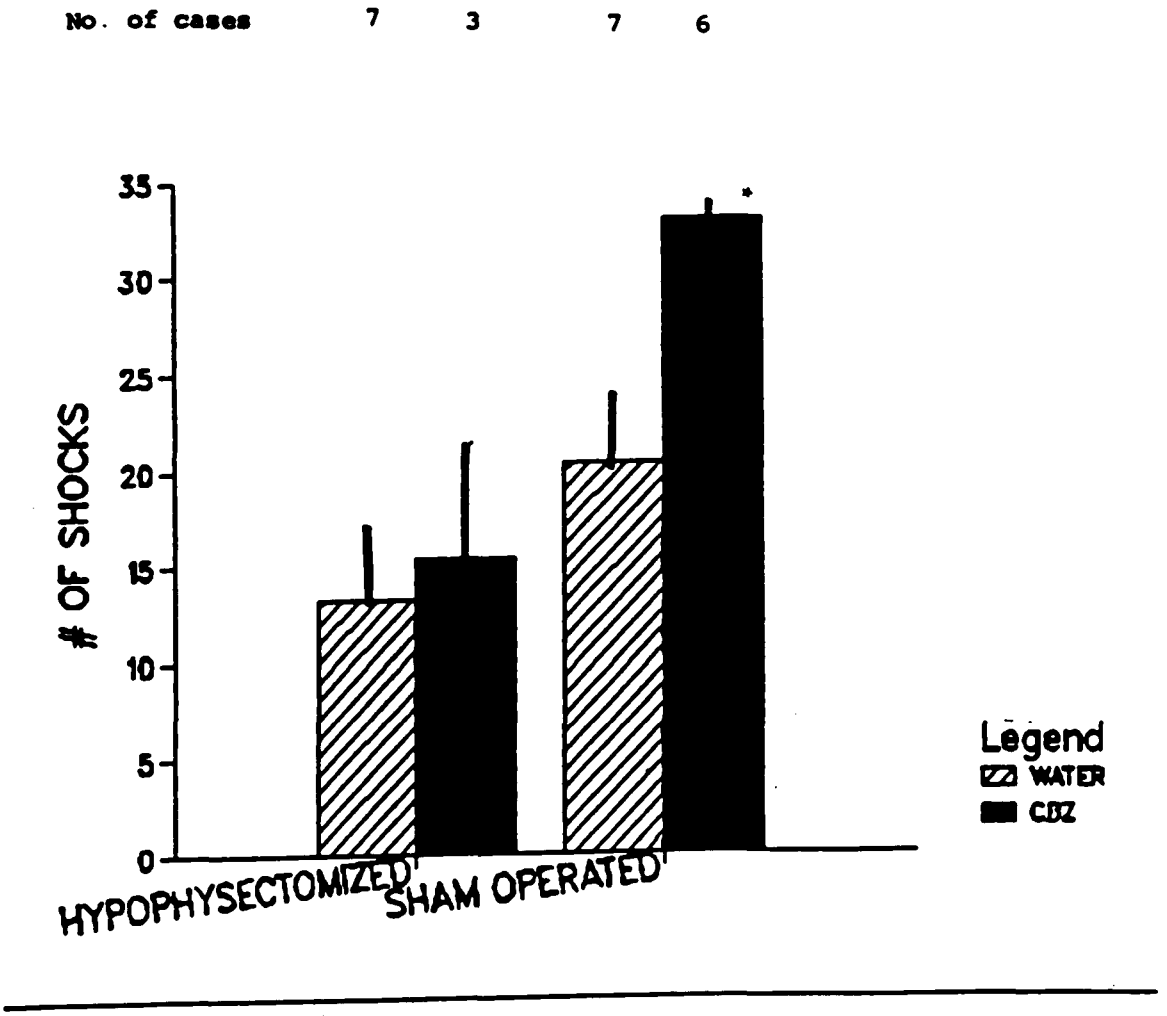
Dose-response relationship of three doses of corticosterone in TRC (Means + s.e.).




* $p<.05$

Figure 13

Effect of adrenalectomy with and without acute chlordiazepoxide administration on responding in TRC (Means + s.e.).




* $p<.05$

Figure 14

Effect of hypophysectomy with and without acute chlordiazepoxide on responding in the TRC. Means and s.e.

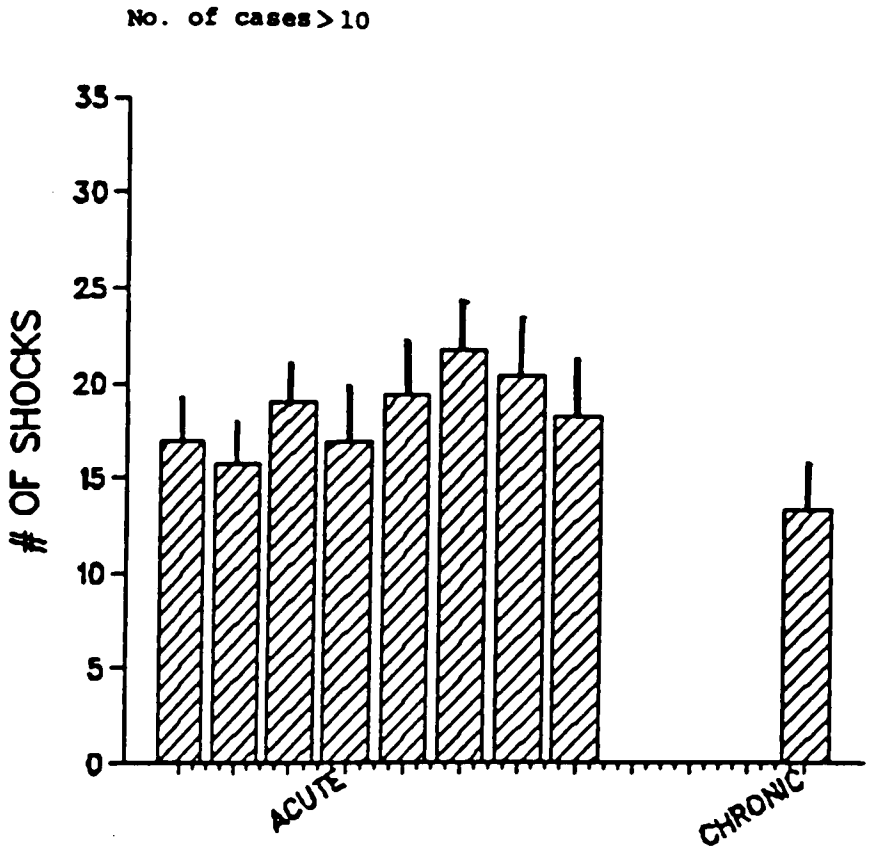



Figure 15

Responding in TRC after acute administration of deionized water to eight groups of animals on eight different days and after chronic administration to one group for five days prior to testing on day six (Means + s.e.).

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