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The behavioral and genetic determinants of anxiety as measured by the effects of anti-anxiety agents in mice

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THE BEHAVIORAL AND GENETIC DETERMINANTS OF ANXIETY AS MEASURED BY THE EFFECTS OF ANTI-ANXIETY AGENTS IN MICE

by

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To my Mom and Dad
who set the following words
to a real life experience...

'Don't walk in front of me,
I may not follow...
Don't walk behind me,
I may not lead...
Just walk beside me and be my friend.'

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Gallaher et al., (1987) have developed two genetic strains of mice through inbreeding, based on their responsiveness to the sedative effects of diazepam. Each of the two strains demonstrated differential sensitivity to the sedative properties of anxiolytic compounds. One strain displayed a reduction in the duration of the sedative effects of diazepam and was designated as diazepam resistant (DR), while the other line was markedly sensitive to these sedative properties and was labeled diazepam sensitive (DS). Gallaher profiled the sedative effects of various benzodiazepines (BDZ) in both strains of mice.

To date, no data has been reported comparing the responsiveness of these mice to the anti-anxiety effects of the BDZ's. Before an accurate assessment of the measurement of anxiety in animals can be made, a suitable method for generating controlled and quantifiable states of experimental anxiety must be developed. For example, can anxiety be experimentally induced in animals? If so, can you objectively measure the effects of anxiety on behavior? It is the intention of this dissertation to critically evaluate existing animal models and develop a method to induce experimental anxiety in mice thus facilitating the measurement, in the DS and DR lines, of possible differential effects toward the anxiolytic properties of BDZ's. If quantitative differences in the anxiolytic response are behaviorally testable then, one can hypothesize that biochemical selectivity should exist for this differential behavioral response. Therefore, these studies will also attempt to correlate biochemical measures of brain BDZ receptor function with behavioral measures of the anti-anxiety effects of the BDZ's, across these two genetically determined lines of mice.

It has been postulated that fear and anxiety are closely associated human clinical states. Darwin (1963) presented the notion of evolutionary selectivity which supports the premise that evolution is in some part reflective of the propagation of the "strong" and the elimination of the "weak" thus favoring the animal with strong adaptive traits. The behavioral response to

the "fight or flight" instinct suggests a strong and controlling place for the experience of anxiety as an "early warning device." Thus, anxiety and fear can be thought to play key roles in animal aggression. If the Darwinian theory is accurate then the characteristic strengths of a species may be genetically coded and passed on from one generation to the next. Based on an evolutionary model, one would assume that if a general behavioral phenomenon such as anxiety exists in humans, some derivation of that behavioral construct should be present in other animals as well. It is not unreasonable to assume that the origins of anxiety first developed in lower animals before being incorporated and modified to meet the demands of human sociological development and progress. Evolutionary similarities, should also exist between the biochemical and neurochemical systems of man and animals. Without this underlying similarity most neuroscientific research would have no vehicle for investigation or baseline for comparison.

In order to develop animal models of anxiety, the relationship between the constructs of anxiety and fear must be well defined. Some (Berger, 1980) have suggested that anxiety is an irrational fear. Others (Greenblatt and Shader, 1974) have described a chronological progression between anxiety and fear and suggest that anxiety is a precursor to fear. Some have noted the similarities in emotional affect but have chosen to distinguish anxiety from fear by the magnitude of the stressful event (Glick, 1976). No one approach precisely explains the intricate relationship between anxiety and fear. Estes and Skinner (1941) operationally defined anxiety as "an emotional state arising in response to some current stimuli which in the past has been followed by a disturbing stimulus." Their definition highlights the survival value of anxiety and its practical significance as a functional early warning device.

One useful approach to understanding anxiety and fear is to place the two constructs at opposite ends of a probability continuum. Their defining characteristics are then a measure of the probable occurrence of a harmful stimulus. Remote stimuli

pose less immediate harm and are therefore anxiety provoking while proximal stimuli that might inflict bodily harm are often feared. An individual's perception of the probability of the occurrence dictates the emotional state. For example, a person suffering from fear of heights, or acrophobia, may become anxious at the thought of climbing a ladder or stepping on the first rung. The probability of being forced to climb the ladder remains low. These thoughts stimulate uncertainty and in turn generate the anxious response. How high will the ladder be? Where will I climb to and when will this occur? The state of anxiety, in response to the anticipation of climbing the ladder, can be modulated into fear by increasing the probability that the stressful stimuli will occur. If the person must climb a twenty foot ladder, to the top of a barn, at 10:00 a.m. the following day, the stressful event will escalate into a feared occurrence. The person will react by either avoiding the ladder or suppressing all responses. Therefore, a stimulus which cues punishment on a variable schedule will induce anxiety and a stimulus which reliably cues the delivery of punishment will be feared. Simple operant conditioning paradigms experimentally induce these conditions. It is possible, then, to develop animal models which successfully induce anxiety and measure anxious responding. These models can be compared to and contrasted with human clinical anxiety. The ultimate goal for these models is to serve as accurate predictors of the human experience and response toward anxiety.

Anxiety can be considered a universal experience. Anxiety includes symptoms involving feelings of unrest and nervousness and may be described by a combination of psychological and somatic complaints. Finally, at extreme levels, anxiety impairs functioning and then can be classified as a disease state. As early as 1941, scientists were developing methods to study the behavioral manifestations of anxiety. Estes and Skinner (1941) developed the first animal model for conditioning anxiety. They described anxiety according to two defining characteristics: (1) an emotional state resembling fear, and (2) the fact that the causal

agents or stimuli neither precede nor coexist with the state of anxiety but are always anticipated in the future. Anxiety then, can be clinically defined as an emotional response to the anticipation of an undesired event which impairs functioning or responding. The severity of the response depends similarly on two conditions: (1) the degree of aversiveness associated with the event, whether real or perceived and (2) the perception of temporal distance between the event and its probable occurrence. As a symptom or syndrome, anxiety may be a normal and appropriate response to a life threatening circumstance. The abnormality in the experience of anxiety exists in proportion to the inappropriate circumstances and inappropriate perceptions of the severity of the anticipated event.

Anxiety can best be understood by separating the manifestation into two principle sub-types. The first is best known as "situational" or stimulus related anxiety. In this case a normally non-anxious person becomes temporarily anxious in response to a set of stressful events. The anxious experience affects the ability of that individual to respond or perform at an otherwise optimum level. The impairment of ability is not directly related to the severity of the anxiety. Instead, an inverted U-shaped function best describes the effects of anxiety on performance. That is to say that low and high levels of anxiety usually have little beneficial effect on performance. Moderate levels of anxiety however, usually enhance performance (Carlson, 1987). Students frequently complain of anxious feelings prior to writing an examination. Businessmen feel anxious when meeting deadlines or when decisions will be made by clients. Professionals are often diffident just prior to a presentation, regardless of their mastery of the material. In each case, moderate levels of anxiety will enhance performance. While the particular environmental contingencies may differ, each description shares with it some degree of uncertainty. It is precisely this element of uncertainty, lack of predictability or simply the absence of control which evokes stress and leads to the anxious experience.

Generalized trait anxiety is the second general type of anxiety. It has been referred to as "free-floating anxiety." In this case, the anxious feelings are usually experienced by individuals as a personality characteristic or a stable and recurring aspect of their life. They are not mediated by specific situational experiences and are often not associated with specific events. The anxiety becomes a manifestation of every day living. The person tends to feel anxious about almost everything. While generalized anxiety is not cued by a specific stimulus or set of stimuli, similar suppression in responding occurs. In studying the etiology of generalized trait anxiety, it is sometimes possible to identify the onset of anxiety with the culmination of a serious event or a serious medical or psychological illness.

Anxiety can be classified based on somatic and psychological symptomatology (Kelly, 1980) as well. Anxiety can generate an array of symptoms ranging from normal every day occurrences to severe distress. Somatic anxiety is frequently expressed by somatosensory manifestations of autonomic functions. These symptoms include tremor, palpitations, tachycardia, dizziness, dry mouth, "clammy" hands, sweating, backaches, neckaches, frequent urination, stomach and bowel discomfort, fatigue, muscular pain, chest pain and headaches. Psychological anxiety refers to internal feelings including: tension, worry, fear, nervousness, irritability and insomnia. Effective diagnostic criteria are imperative in evaluating specific treatment modalities. Recent attempts have been made to standardize the diagnostic criteria used for anxiety. The American Psychiatric Association has published guidelines on the criteria and differential diagnosis for anxiety disorders. These operational definitions are listed and described in the Diagnostic and Statistical Manual of Mental Disorders (DSM III, 1987). DSM III subdivides anxiety into: (1) generalized anxiety, (2) simple and social phobias and (3) panic disorders. Generalized anxiety is typically referred to by the term "anxiety" with phobias and panic attacks as specific subsets. For purposes of this discussion, references made to "anxiety" will refer exclusively to "situational" anxiety.

Once an accurate diagnosis has been made for an individual suffering from anxiety, certain treatment decisions can be implemented. Anxiety, as previously mentioned, can vary in intensity. For some, moderate anxiety is productive and motivates behavioral output. Mild anxiety is usually detectable by the existence of relatively few somatic symptoms. The person may complain of nervousness or other related psychological symptoms but usually will not suffer from somatosensory manifestations. The key in assessing the need for clinical intervention is a subjective appraisal of the quality of the patient's life. If it is apparent that the patient is suffering from situational anxiety caused by specific sources and if the patient is functionally impaired, treatment is usually prescribed. In this case, the quality of living is negatively affected by the anxious behavioral state. Emotional support and psychotherapeutic counseling are frequently effective in helping the patient link the symptoms to specific circumstances. Psychiatric intervention is less likely to be effective for the patient who is unable to link the symptoms with causal events.

The pharmacotherapy employed in treating the symptoms of anxiety are represented in the class of compounds identified as the "anxiolytics." Anxiolytics, by definition, decrease anxiety. The benzodiazepines (BDZ) are by far the most widely recognized and prescribed drugs of this class. The barbiturates are grouped under this category as are meprobamate and alcohol. Although the barbiturates were often prescribed to treat the symptoms of the "anxious patient", meprobamate was the first chemical agent used for the sole purpose of reducing anxiety (Hill et al., 1971). Barbiturates and meprobamate produce serious side effects resulting in tolerance after extended use and severe symptoms upon withdrawal. In addition, clinically effective doses of some barbiturates are not substantially removed from doses which cause respiratory depression (Hill and Tedeschi, 1971). The BDZ's replaced these compounds as the drugs of choice in treating anxiety, mainly because of their increased efficacy and relative safety in clinical treatment.

The behavioral effects of the BDZ's can be described as disinhibiting. To borrow from previous examples, if the anxiety is pathological, the anxious feelings experienced by a student toward an expected exam or the professional toward the anticipated presentation will in all likelihood produce escape behavior or suppression of appropriate goal directed behavior. In a wide variety of behavioral situations, anxiolytic agents can be shown to increase suppressed baselines. In addition to their antianxiety effects, the BDZ's also produce: (1) sedation, (2) muscle relaxation, (3) anti-aggression, (4) anticonvulsant activity, (5) analgesia and (6) potentiation of the depressant effects of ethanol. The BDZ's are also used in the treatment of alcohol withdrawal and certain gastrointestinal disorders. Over the past ten years, the investigation of BDZ's has become one of the most aggressive efforts in molecular neuropharmacology. The BDZ's are considered, by the medical profession, to be the drugs of choice for the pharmacotherapy of anxiety, sleep disorders, emotional disorders relating to anxiety and some convulsive states (Petersen, 1983). In addition, they are administered as centrally acting muscle relaxants and as inducing agents in anesthesia. Over the past twenty years research efforts have developed some thirty BDZ compounds approved for clinical use.

The ongoing quest for new anxiolytic compounds with fewer side effects has led to a proliferation in the development of potential anxiolytic compounds. A need has arisen for the development of valid and reliable animal tests to evaluate these compounds. Animal models have been used classically to predict the safety and clinical efficacy of potential novel agents (Cook and Davidson, 1973). Obvious dangers in testing unknown compounds on humans have reinforced this approach. Animal models also afford the neuroscientist the ability to test the biochemical mechanism of action and relate the behavioral effects to neurophysiological and neurobiochemical events. A great deal of precision and control are necessary in order to correlate animal behavior and biochemical drug related effects with clinical efficacy. One compelling concern of anxiolytic testing is whether

adequate face and predictive validity can be demonstrated among animal models, biochemical measures and human clinical anxiety.

It is important to evaluate the criteria used to develop animal models which are employed to evoke, measure and correlate pharmacologic effects of anxiety between animals and man. The task of evaluating animal models is not without its share of ambiguity and complications. The problem can be simplified by highlighting three basic approaches by which the adequacy of a given animal model can be judged. For purposes of discussion they will be referred to as "correlate, symmetry and restriction" models. The correlative approach (Carlton, 1978) is frequently cited in the literature with ample justification. This approach incorporates the necessary elements for describing the predictive validity of a given animal paradigm. The predictive validity addresses the issue of comparison and predictive strength between data generated from a given animal model and the pharmacologic response in humans. It is the central focus of any model since ultimately the data must predict the clinical response of a given class of agents. In order to show predictive validity, the correlative model must address three basic issues (Glick, 1976). First, the animal model must generate dose response profiles for standard anxiolytic agents, known to reduce clinical anxiety. Second, the model must identify relative potencies in animal testing which correspond with human clinical experience. Third, the animal model should display a high degree of selectivity toward a given therapeutic class of agents. For example, the animal test should be able to distinguish the effects of nonanxiolytic agents from known anxiolytic compounds.

The correlative approach does not require any demonstration of face validity. Face validity is a construct which describes the logical correspondence between a response measured in animals and an analogous clinical response. For example, inhibition of pentylenetetrazol (PTZ)-induced seizures is a reliable initial screen for agents which show anxiolytic activity in humans (Swinyard and Castellion, 1966). There is no logical concurrence however, between seizure activity in mice and

anxiolytic activity in humans. This example demonstrates low face validity. As long as the methodology yields "correlative" effects, no further justifications are needed. There are, however, certain problems which may occur when conditions of face validity are ignored.

"False negative" is a term referring to the chance that a given animal test will overlook a potentially active compound. This becomes a realized occupational hazard associated with the correlative approach. For example, a compound might possess strong anti-anxiety properties without showing anticonvulsant effects. Testing for the inhibition of PTZ-induced seizure activity would be inappropriate and would not identify this compound for further verification. The converse problem is referred to as "false positives". These are compounds which might test positive in an anticonflict test but in actuality show no anxiolytic effects. The potential damaging results of false positives is of lesser importance. If an agent were to test positive on a preliminary screen, supporting evidence would be warranted. Additional screens and the possibility of human clinical trials would most certainly highlight any lack of significant anxiolytic effects. In contrast, the likelihood of further testing a "false negative" is slim and can result in a serious oversight.

The symmetry model adjusts for face validity in generating some coefficient of parity between the animal test and the construct for which it is testing. It is not simple to correlate animal and human behavior and thus the development of symmetrical models becomes a challenge. An isolated physiological response such as blood pressure or seizure activity, as measured respectively by manometric and electrophysiological techniques, is not a difficult comparison to be made between animals and humans. Both species can be objectively measured using these techniques and the results can be directly compared. Measurements of anxiety are not as easily quantified in animals or man for that matter. The necessary measurement often requires creative behavioral manipulation and extensive training. PTZ-induced seizure activity provides an excellent example of the

advantage of "symmetry" in animal models. The PTZ test describes the agent's ability to effect seizure activity. Therefore, it would make "good sense" to test this construct against these experimental conditions. While the PTZ test fulfills all the criteria of the "correlative" approach, it lacks logical predictive relevance toward anxiolytic effects.

The "restriction" model qualifies behavioral intent. The manipulation of certain variables produces control over the net results. In order to control for differences in behavior, one restricts the context or the antecedent conditions, which generate the behavior under investigation. The investigator operationally defines the response in terms of the response measurements. For example, an investigator may be interested in measuring the effects of anxiety on social activity in rats. The researcher chooses a restrictive model by identifying specific, isolated and observable social responses and measures them against the effects of aversive stimulation. These restrictions facilitate the comparison between animal responses and clinical states.

Each of the three models, correlate, symmetry and restrictive, are alone imperfect. Their relative shortcomings are apparent when each is considered separately and independent of the others. It is fortuitous to consider models which incorporate the strengths of each while at the same time compensate for their individual weakness. The criteria which will be employed to critically evaluate the existing animal models of anxiety will be interactive. First, the model must generate predictive validity in the ways described in the correlative method. Second, the model must incorporate some logical progression from the investigated construct to the measurement device and response selection. Third, some specificity in response control is necessary in order to draw similarities between animal and human response repertoire. Given that a method demonstrates adequate correlative merit, it can then be judged on constructs of face validity.

Researchers have created two categories of behavioral paradigms in testing anxiolytic drug action in animals. The first class is comprised of models which test the effects of anxiolytics

on unconditioned animal reactions. The unconditioned response methodologies which will be reviewed include: PTZ-induced seizure activity, somatic stress, consummatory behavior, exploratory and social behavior. The second category of animal models is based on animal learning paradigms. These profiles include: conditioned emotional responding, conditioned avoidance and punished behavior. Each will be judged on the merits of their correlative effects (i.e. dose dependency, sensitivity, potency and selectivity) and the strengths in demonstrating adequate face validity.

The inhibition of PTZ-induced seizure activity is often used as an initial screen in testing anxiolytic activity of drugs in rodents (Hill and Tedeschi, 1971). The test is very sensitive to a wide range of anxiolytic agents. In addition, the relative potency of the anxiolytics necessary to successfully block induced seizure activity corresponds to the magnitude of their anxiolytic effects in humans (Childress and Gluckman, 1964). Other agents such as antiepileptics can be distinguished from anxiolytic drugs by the specificity of the convulsant response. In the case of diphenylhydantoin, an antiepileptic, tonic seizures are inhibited. While a few anti-convulsant agents do test positive on this measure, the test is moderately selective (Irwin, 1967). The sensitivity and speed of this measure account for the the popularity of the PTZ model in initial anxiolytic screening. Large numbers of animals are, however, necessary in order to show dose response differences. One drawback, which has been previously discussed, is the lack of convincing face validity. Present knowledge of physiological mechanisms does not support any correlation between human anxiety states and convulsive mechanisms in animals. Conditions of experimentally induced somatic stress have been evaluated as possible screens for anxiolytic agents. Anxiolytics have been documented to antagonize stress associated with somatic variations. Elevation in blood pressure (Benson et al., 1970) and body temperature (Delini-Stula, 1971) have been explored as measures of somatic stress. Certain experimental conditions have been implemented

including forced swimming (Le Fur, et al., 1979) and electric shock (Bassett and Cairncross, 1974) as stimuli which reliably produce somatic stress. Certain anxiolytics do indeed antagonize these stress reactions in a dose dependent fashion with a fair degree of sensitivity. However, the antagonism is not restricted to anxiolytics. The physiological effects of somatic stress can be blocked by antidepressants, neuroleptics and some anticonvulsants. (Keim and Sigg, 1977). Measures of somatic stress are not selective and therefore this model has not won overwhelming approval or widespread use as an initial screening technique for anxiolytic activity.

Initial behavioral tests on the benzodiazepines noted a particular, and consistent biobehavioral effect. The BDZ's were reported to increase consummatory behavior in experimental animals (Randall and Kappell, 1961). Cole (1983) presented conclusive evidence to support these contentions. Rats pretreated with chlordiazepoxide were tested for increases in consumption under conditions of satiation and deprivation. Both conditions resulted in similar increases in consumption. Additional evidence was presented on the differential effects toward novel verses familiar foods. Consumption increased for both conditions with a higher magnitude of effect demonstrated in response to novel foods (Paschel, 1971). At first glance, novel foods would not appear to be an aversive stimulus. If the digestive system of the rat is carefully reviewed, it is apparent that it possesses no regurgitative abilities. If a rat ingests a novel harmful substance, extreme discomfort and even death may ensue. Eating behaviors of rats are affected by these constraints. Rats exposed to a novel substance (Garcia and Koelling, 1966), under non-drugged conditions, will cautiously ingest small amounts. If no adverse physiological effects are experienced they may continue eating. Benzodiazepines may reduce the aversive component of the food stimuli and decrease suppression toward a higher intake of novel and possibly dangerous substances. Two other parametric variations were studied as well. Soubrie et al. (1976) studied the effects of novel verses familiar environments on BDZ increases in

consumption. No statistically significant differences exist for either environmental condition.

BDZ modulation in consummatory behavior was tested in various species in order to ascertain whether this effect was species specific or a general phenomenon. Studies were run in rats (Cooper and Francis, 1979), dogs and cats (Della-Fera et al., 1980), hamsters (Birk and Noble, 1981), pigeons (Cooper and Posadas- Andrews, 1979), horses (Brown et al., 1976) and humans (Tobin and Lewis, 1960). Based on extensive studies and the conclusive data collected, it is understandable that consummatory behavior has been identified as a measurement tool for anxiolytic drug action. The dose response functions and potencies generated from these studies are comparable to those used to treat clinical anxiety. Interestingly, one of the noted clinical side effects of the BDZ is increased consumption (Sepinwall and Cook, 1978). These studies show high selectivity for the BDZ's with no effects observed for non-anxiolytic compounds such as anticonvulsants, antidepressants or neuroleptics. However, certain known anxiolytics (meprobamate) do not increase food intake (Pollard and Howard, 1979).

Modulation in exploratory behavior has also been evaluated as a viable construct for testing the anxiolytic properties of agents. In unfamiliar environments, anxiolytics tend to increase locomotor behavior in rodents (Christmas and Maxwell, 1970). The effect is quite selective and does not respond to antidepressants, anticonvulsants or neuroleptics. This data would suggest some significant degree of predictive validity. However, the mechanism of action is complex. The stimulatory effects of the anxiolytics on exploratory behavior appear biphasic (Marriott and Smith, 1972). The drug, at low doses, produces an increase in exploratory behavior. At high doses, however, the response is inhibitory. To further complicate the issue, these effects are also time dependent. Animals show increases in exploratory behavior early in the test session but one might say the "novelty wears off" and the animals may show inhibition of exploratory behavior later in the test period (Iwahara and Sakama, 1972). It has been

documented (Itoh and Takaori, 1968) that anxiolytics will increase exploratory behavior in novel environments. The decrease in response, over time, may be reflective of the rodents cumulative experience and familiarity with the environment.

Measuring anxiolytic effects against exploratory behavior, as it exists, does not fulfill the criteria for adequate predictive validity. Furthermore, it is not clear that these anxiolytic agents are selectively increasing exploratory behavior. More compelling evidence is presented by Kumar (1971). In a set of experiments designed to ascertain the facilitory affects of chlordiazepoxide on exploratory behavior it was shown that rats, previously exposed to electric shock, did not differ appreciably on measures of exploratory behavior when compared to rats never exposed to shock. According to the known anxiolytic effects of chlordiazepoxide, the shock experienced rats should have demonstrated higher levels of exploratory behavior as a result of decreased levels of anxiety associated with prior exposure to the negative stimuli. As it appears, this measure is not selective or sensitive to fear induced conditions and may instead be measuring non-specific total increase in motor output.

Certain modifications have been introduced to the exploratory model, increasing its predictive success. Crawley and Goodwin (1980) developed a model based on the natural tendency for rodents to avoid brightly lit areas. The test apparatus consisted of two adjoining test chambers. The animal's natural response to the brightly lit chamber was to escape into the adjoining dark chamber. The number of transitions from light to dark were recorded. This measure generated broad dose response profiles for anxiolytics such as diazepam, meprobamate and pentobarbital. Rodents are nocturnal animals and therefore will prefer dark to light. Light then can be defined as an aversive stimulus, dictating punishment-like effects. The anxiolytics decrease aversion toward light and in so doing, decrease the number of transitions from light to dark. Total motor increases were also evidenced, in a dose response fashion, for the same anxiolytics. Non-anxiolytic compounds tested in this procedure

did not show similar effects. This procedure can be expected to identify the effects of stimulants as well and would therefore, confound anxiolytic testing. In order for this test procedure to be properly validated, the differential effects of stimulants and the anxiolytics would need to be addressed. While this data is not available, major confounding effects are anticipated in validating a method involving motor output against the criteria of selectivity toward non anxiolytic compounds.

A social activity measure (File, 1978) was developed in part to circumvent some purported problems associated with conflict procedures. According to File (1978), evidence suggests that deprivation conditions can alter an animals response motivation. In addition, intraperitoneal administration of compounds can affect the animals sensitivity to painful stimuli (electric shock). For these and other similar concerns, File developed an unintrusive measure of anxiety. The procedure manipulates light intensity and familiarity with the testing environment. The animals were scored according to the manifestation of the following behaviors: sniffing, following, grooming, kicking, mounting, jumping, wrestling and crawling under or over the partner. Social activity decreases when the rats are either unfamiliar with the environment or the lights are brightly lit. File (1980) has demonstrated that administration of chlordiazepoxide increases social activity in a dose dependent fashion, under these conditions.

Certain confounding effects are apparent with this methodology. It is difficult to identify any one social response which is not somehow related to other such behaviors. Social interaction does not specify a set of separate behavioral events but instead can be understood as the relationship between and among a given set of behaviors. Furthermore, it is difficult to justify the relevance of social activity to the construct of anxiety. It is equally difficult to relate these findings in some way to similar human experiences which constitute anxiety-like effects. Correlative studies have also suggested that social activity measures are responsive to extraneous variables (File and Hyde,

1978). The weight of the animal and the time of day during which they were tested significantly affect these measures of social activity. Certain anxiolytics increase social activity in the absence of aversive stimuli and suggest a non-specific increase in social behavior (File and Hyde, 1979). Finally, activity measures are confounded by false positive results when testing certain non-anxiolytic compounds, such as stimulants. Therefore, while this measure does highlight potential methodological consideration in profiling anxiolytic effects of compounds, these confounding factors must be eliminated in order to present an accurate predictive and correlative model of animal anxiety.

Traditional conflict protocols are a popular method for testing the efficacy of anxiolytic agents in animals. It is believed that employing well trained animals will decrease the variability within the experimental system (Sepinwall and Cook, 1978). Researchers have invested a great deal of confidence in the degree of face validity demonstrated by the learning paradigms. The concordance existing between the aversive stimuli (shock) and the response (suppression) is logical and can be associated with human clinical anxiety. It is relatively clear that the anticipated aversive physical effects of shock in animals can be likened to the perceived remoteness of physical harm experienced clinically. This section will seek to explain the merits of testing the effects of anxiolytics by traditional learning paradigms.

The effects of punished behavior in animal models of anxiety have been extensively investigated (Sepinwall and Cook, 1978). Punishment can be operationally and contextually defined as the presentation of aversive stimuli which suppresses otherwise trained, reinforced responses. A classic operant model typically conditions bar pressing and a food reward. In order to suppress the conditioned response, foot shock is paired with the delivery of the reinforcer. The animal inhibits responding during the likelihood of the delivery of aversive stimuli and potentiates responding during otherwise free periods. This procedure represents the basic operational construct developed in the Geller conflict test (Geller and Seifter, 1960) and other similar models

(Vogel et al., 1971). The results generated from these models are valid and reliable predictors of anti-anxiety behavior and anxiolytic drug action. Operant conflict models train animals to bar press, according to a schedule of reward. The schedule is typically divided into two segments, free and punished. Initially the animal is trained according to a variable schedule of reinforcement, usually involving a consummatory reward. Once the acquisition of the response is generated, the second segment of the conflict schedule is introduced. This segment always follows the first and is signaled by an external cue. The animal, now trained to respond, is reinforced and punished simultaneously with food and electric shock. After repeated training sessions, the animal learns to decrease responding during the cued punishment period and increase responses during the safe interval. Inhibition of the animal's punished responses is a strong measure of face validity and strongly correlated with clinical anxiety. Thus a good deal of isomorphism can be seen between the animal's inhibition in responding, during anticipated periods of shock and the anxiogenic suppression of human behavior.

Predictive validity has also been demonstrated. When trained animals are injected with benzodiazepines, their rate of responding increases during punishment periods with little or no change in free responding. Nonanxiolytic agents such as neuroleptics, antidepressants, stimulants or analgesics either have no significant effect on punished responding or inhibit responding further from baseline (Sepinwall and Cook, 1978). Conflict procedures have been profiled on a variety of species including pigs (Dantzer, 1978), cats (Jacobsen, 1957), pigeons (Witkins and Barrett, 1981), monkeys (Patel and Migler, 1982) and humans (Beer and Migler, 1975). The effective relative potencies in these species correlate strongly with clinical potency. Their demonstration of excellent selectivity, dose-response potentiation and sensitivity toward anxiolytic agents fulfill the criteria for valid behavioral testing of anxiolytic drug effects in animals.

The effect of anti-anxiety compounds in conflict models depends on prior experience or presentation with the drug in question. First time administration of an anxiolytic will not necessarily show a potentiation in responding during the punishment period. Margules and Stein, (1968) presented data which suggested that the animal's first experience with anxiolytics, such as diazepam and chlordiazepoxide, often increase suppression in punished responding, which is different than the response from subsequent treatment. Accordingly, anxiolytic produced increases in punished responding does not occur prior to several administrations and in some cases a decrease in punished responding is produced. This initial lack of effect is thought to be due to the sedative effects of the anxiolytic agents. In conflict measures the sedative effects tolerate while the anxiolytic effects are maintained with repeated treatment. These sedative effects in animals and man show tolerance and may be related to the delayed onset of effect of anti-anxiety activity in man. Other sedative agents (neuroleptics, antihistamines and anticholinergics) do not show broad antianxiety activity in man (Rickels et al., 1978). It is fair then, to assume that sedation is not a necessary component of the anxiolytic response.

Judging from the information reviewed in the previous paragraphs, conflict procedures provide the best means for testing anxiolytic activity of drugs in animals. Most animal conflict models regardless of "contextual camouflage" measure aversive control over responding. Given the choice, it is advantageous to choose a measure which directly controls the parameters of the aversive stimuli. Conflict measures allow the investigator to have explicit control over the magnitude and the delivery of the aversive stimuli. These controls are not realized in exploratory models or social activity measures. Neither allow specific modulation of aversive intensity or response contingent control over the stressful stimuli. For example, a novel environment or brightly lit area cannot be adjusted based on the animal's response. They are simply presented on a single trial basis and the animal's response recorded. No baseline recordings are

available. The animal's performance is compared only to a control group comprised of different animals. Variability in intersubject samples is always higher than the variability between intra-subject groups. Operant conflict procedures employ training which is conducive to generating strong and stable baseline recordings.

Conflict models are excellent predictors of clinical anxiety. Few would argue that the demonstrated face and predictive validity incorporated in these paradigms, is matched by any of the other animal models of anxiety. While some models boast of anxiolytic selectivity, few can distinguish without exception an anxiolytic from a non-anxiolytic agent. The motor related measures, such as social activity and exploratory behavior, are sensitive to stimulants. The response to anxiolytic and stimulant compounds is similar and points to the notion that these models are not measuring anxiolytic effects but instead measure motor output. The consummatory models are confounded by the possibility that certain compounds, regardless of anxiolytic activity, increase physiological drives associated with eating and drinking. Conditioned emotional responding and conditioned active avoidance are equally problematic. In their case neither is successful in generating selectivity, sensitivity or correspondence to clinical anxiety.

The conflict models not only generate selectivity and sensitivity in dose response profiles but correlate to clinical treatment phenomena as well. The longer acting benzodiazepines (diazepam, chlordiazepoxide) on average tend to accumulate with steady state levels increasing for up to one to two weeks of administration. Clinically, this accumulation corresponds with a gradual increase in anxiolytic effects. Margules and Stein (1968) provide data to support that first time administration of known anxiolytics in conflict procedures does not increase punished responding. At first glance, these results would seem counterintuitive. In actuality, they are correct and support the evaluative power of this model. Acute administration does not resemble the clinical administrative regime for the anxiolytics. It

is highly possible that the model is initially identifying the sedative effects of the BDZ's which antagonizes their effects on punished behavior over time. The animals tolerate to the sedative effects of the drug but the antipunishment effects are not compromised. The combination of controlled baseline responding, control over delivery and magnitude of aversive stimuli, excellent face and predictive validity and the requirement of relatively few animals make the conflict model the preferred method of choice in anxiolytic testing.

From a purely practical vantage point, a repeat measure conflict procedure decreases the number of animals needed for significant results. In procedures such as the Vogel et al. (1971) conflict model, large numbers of animals are necessary in order to generate broad dose response profiles. The Vogel thirsty rat conflict procedure employed the operant response, licking, as a measure of the anti-anxiety effects of anxiolytic agents. The animal was placed in the apparatus 30 min. after interperitoneal injection with selected doses of a known anxiolytic. The animal was allowed to find the drinking tube and complete 20 licks before shock was administered. A timer automatically monitored a three minute interval during which shock was delivered following each twentieth lick. The number of shocks delivered were recorded for each subject. The animal was exposed to this conflict paradigm only once, since an animals' familiarity with the test condition would confound the results. This conflict model generates dose response data for anxiolytic drugs which correlates highly with clinical efficacy (Clody, Lippa and Beer,1982). While an efficient model, such as the Vogel paradigm, can swiftly profile a large number of drugs, it requires a vast number of animals. When conditions limit the availability of large numbers of animals, such as in studies of specially selected mice, repeated measures conflict procedures become a necessary alternative.

Gallaher, et al., (1987) demonstrated behaviorally, in DR and DS mice, the sedative effects of known anxiolytic compounds. In order to confirm these results, an independent measure of sedation will be investigated. The effects of ethanol will be

profiled as an independent measure of sedation. In addition, the potentiation of the sedative effects of ethanol by a benzodiazepine will also be investigated in order to generate yet another independent measure of the behavioral response, in these divergent strains, to the sedative effects of the benzodiazepines.

The pharmacological profile of ethanol is similar to the BDZ's. The similarities in responsiveness account for some apparent anticonflict activity, under limited experimental conditions. Sufficient evidence exists from behavioral and electrophysiological studies which indicate that ethanol effects the central Gabaergic mechanisms. Liljequist and Engel (1984) studied the comparative anticonflict effects of diazepam and ethanol. Intraperitoneal administration of 2.5 mg/kg diazepam and 3.0 g/kg ethanol were separately profiled using a modified Vogel et al., (1971) conflict design. The dose response functions were similar with equi-effective anxiolytic doses realized for each of the two compounds tested. Neither drug condition differed along the measure of the number of shocks taken during the punishment period. The authors stress that ethanol has a narrow margin for effective anticonflict activity without inducing sedative effects. A dose of 3.0 g/kg ethanol was optimal in producing anxiolytic effects without marked sedation. Doses of ethanol exceeding 3.0 g/kg produced sedation while doses lower than 3.0 g/kg were effectively tolerated by the animals and demonstrated little anxiolytic effects. Bicucculline, a GABA antagonist, administered intraperitoneally at a dose of 1.0 mg/kg, 30 minutes prior to conflict testing, did not effect anticonflict activity induced by ethanol or diazepam. However when picrotoxin was administered at a dose of 1.0 mg/kg, under the same pretreatment conditions, complete antagonism of the effects of ethanol were produced with no significant change in diazepam responding. These data suggest different neurochemical mechanisms active in the anticonflict effects of these two drugs. One might consider that the anticonflict effect mediated by ethanol may be produced by an interference in central Gabaergic activity resulting from a picrotoxin sensitive receptor site. This description of mechanism

is based on hypothetical deductions made from available biochemical data.

Based on the information reviewed above, the conflict model can be regarded as a selective, clinically relevant model for testing the effects of anxiolytic compounds in animals. This dissertation will profile the behavioral effects of known anxiolytics on conflict measures in drug resistant and drug sensitive mice. The training and testing schedules borrow from the Geller/Vogel conflict protocol for rats with appropriate modifications made for testing mice. Therefore, the ease of training a naturally occurring response (licking), consistent baseline performance and multiple schedule fixed ratio punishment should allow the development of a reliable measure of the effects of anxiolytic agents in these two divergent strains of mice.

BIOCHEMICAL EFFECTS:

Receptors are membrane bound proteins responsible for the recognition of hormone or drug molecules, transducing a signal across the cell membrane, initiating a biochemical response and ultimately a physiological response. Receptors were first recognized by J. N. Langley (1905) as physiological entities directly responsible for drug effects. Clark (1937), along with others, described the competition of agonists and antagonists for specific receptors in isolated tissue preparations. The concept of receptors has since evolved from these early hypotheses of hormone and drug action to the recent isolation, purification and crystallization of certain receptors in addition to cloning of the genes responsible for receptor protein biosynthesis.

As refinements were being made to the behavioral screens for anxiolytic drugs, several neurochemical mechanisms were proposed to explain the various actions of the BDZs. Among the systems studied were glycine receptor sites (Young, Zukin and Synder, 1974), serotonin (Geller and Bloom, 1970) and cyclic AMP (Beer et al., 1972). In 1974, however, Haefely et al. (1975) and Costa et al. (1975) demonstrated that the BDZs interacted specifically with one neurotransmitter system, the GABA system. Several years later a recognition site for the BDZ's was demonstrated in the CNS tissue (Mohler and Okada, 1977; Squires and Braestrup, 1977; Mackerer, et al., 1978). The ability of BDZs to displace radiolabeled ligand from these sites correlates very well with their clinical efficacy (Mohler and Okada, 1977). To this date, the BDZ receptor remains the most plausible model for investigating and explaining the biochemical correlates to the behavioral pharmacology of anxiety and therapeutic intervention.

Brain BDZ receptor sites are located primarily on cell neurons and are enriched in synaptic membrane preparations (Mohler and Okada, 1977), a finding consistent with a neuromodulatory role for these agents. BDZ receptors are unevenly distributed in the brain (Braestrup and Nielsen, 1983) indicating that BDZ binding is not a non-specific membrane

phenomenon. Autoradiographic studies (Young and Kuhar, 1981) have demonstrated that this regional distribution of the brain BDZ receptors is remarkably consistent among various species. In mice, the largest concentration of BDZ receptors is present in the cerebral cortex and cerebellum. The next highest concentration of BDZ receptors is found in the hippocampus (Mohler and Okada, 1978).

As mentioned earlier, the inhibitory neurotransmitter GABA has been functionally linked to BDZ interaction (Costa et al., 1975; Haefely et al., 1975). The BDZ's facilitate GABA mediated transmission at pharmacologically relevant doses and thus potentiate GABA-mediated inhibition in the CNS (Liljequist and Engel, 1984). It has also been reported that GABA potentiates BDZ binding (Tallman et al., 1978). This enhancement in BDZ binding is caused by an increase in BDZ receptor affinity for its ligand with no change in the number of receptors. This effect is specifically blocked by the GABA antagonist bicuculline. The magnitude of GABA potentiated BDZ binding is region specific and can be explained by either heterogeneity in the BDZ receptors (Squires et al., 1979), the GABA receptors (Braestrup, et al., 1979) or by a variable interaction between the two binding sites.

Anions such as chloride, bromide, iodide and thiocyanate also enhance the binding of BDZs by regulation of receptor affinity (Costa et al., 1979). This observation indicated that the BDZ receptor might be functionally coupled to an ionophore. Chloride interacts directly with BDZ or GABA binding sites (Braestrup and Nielsen, 1983) supporting the hypothesis that these receptors are somehow coupled to a chloride channel. It is now well established that the chloride channel functions as an effector in the transmission of BDZ receptor effects. Both GABA and BDZ agonists have been reported to potentiate the opening of the chloride ion channels. Clinically active BDZ's do not affect conductance within the ion channel but instead, increase the frequency of channel openings. In addition, these active BDZ compounds also increase the duration for which the channel remains open.

The BDZ, GABA recognition sites and a chloride ionophore have been incorporated into what is referred to as the supramolecular complex (see Appendix 1). In this schematic, (Haefely, Kyburz, Gerecke and Mohler, 1985) the complex is pictured as a tetramer. Each of the four monomers contain binding domains for GABA and the BDZs. The pore created by the association of the tetramers is the chloride ionophore which contains a binding domain for BDZ's, GABA, barbiturates and convulsants. In this depiction, the largest arrow indicates the opening of the channel by the binding of GABA to its receptor; the BDZs allosterically modulate this GABA-channel interaction as indicated by the second largest arrow; modulation of the channel by barbiturates and convulsants is indicated by the small arrow. Ligand-induced conformational changes in any one of the three binding domains is capable of inducing changes in either of the remaining two as indicated by the bidirectional arrows in subunit four.

The recent development of radioactively labeled compounds with high specific activity has permitted the study of the biochemical mechanisms of action associated with the BDZ receptors. Radiolabeled BDZ's (tritiated ligands) are incubated with tissue samples rich in BDZ receptor populations. The ligand binds to the receptor site in a process driven toward equilibrium. Once the receptor is labeled it is possible to quantitatively determine the number and affinity state of the BDZ receptor sites present. This method has been successful in demonstrating the presence of high affinity binding sites for brain BDZ receptors. These sites also demonstrate saturability, selectivity and reversibility. Since a finite number of receptors exist for each cell, one would expect to generate a saturation isotherm which at some ligand concentration reveals saturability of all existent receptor sites. Similarly, BDZ receptor binding should demonstrate specificity to BDZ (and anxiolytic) compounds and not respond to other transmitters or compounds outside this pharmacological classification. Finally, since most drugs and neurotransmitters function in a reversible manner, it follows that BDZ binding should

itself be a reversible process. Therefore, it should be possible, by inclusion of excess unlabeled ligand, to dissociate the radiolabeled ligand from the receptor site.

Rates of association and dissociation are measures of the affinity of the receptor for a ligand. Based on the law of mass action, the binding of a ligand to a receptor can be described as follows: $L+R \Leftrightarrow LR$. The rate of association (K_a) can be described by the preceding equation. It is a simple measure of the attraction of the ligand for the receptor sites. The K_d or dissociation rate constant is a measurement of the degree of difficulty involved in separating the ligand from the bound receptor. The K_d can be described by reversing the previous association equation: $LR \Rightarrow L+R$. Based on these two equations it is possible, by mathematical manipulation, to calculate the affinity constant for a particular ligand and receptor population. The affinity constant is a quantitative estimate of the physical interaction between drug compounds and respective receptor populations. These kinetic measurements are used to characterize receptor populations and the biochemical pharmacolgy of the ligands (neurotransmitters or drugs) that bind to them. This data may then be correlated to neurochemical, physiological and behavioral responses.

In vitro receptor binding thus generates pertinent data in profiling the structure and activity of the BDZ receptor ligands. In vitro studies allow one to study receptor binding and the resultant biochemical effects without the interference of neighboring physiological structures. [^{3}H]flunitrazepam is the ligand of choice when studying in vitro BDZ binding due to its slow off rate as determined from dissociation studies (Chang and Synder, 1978). Dissociation experiments routinely calculate this rate by the inclusion of a highly concentrated solution of cold ligand. The disproportional concentration of cold to hot ligand displaces binding of the radioligand from the receptor.

As mentioned previously, BDZ receptor binding studies (Haefely et al., 1981) have demonstrated an allosteric relationship between GABA (an inhibitory neurotransmitter) and the BDZ's. When GABA is present in [^{3}H]diazepam binding assays, BDZ

receptor binding increases for agonists but not antagonists such as Ro 15-1788. No change is measured in the total number of available BDZ receptor sites. However, a change in receptor affinity (K_d) is evident. This increase in binding is a direct result of an increase in the affinity of the BDZ receptor for the BDZ's (Tallman, et al., 1978). This potentiating effect of GABA can be blocked by specific GABA antagonists such as bicuculline. These data support an allosteric interaction between the GABA recognition site and the BDZ recognition site. Braestrup and Nielsen (1981) developed an assay to test the effects of GABA on BDZ binding. According to their data, the enhancement of BDZ binding in the presence of GABA occurs only for those compounds which are agonists at the BDZ receptor site. Binding of BDZ antagonists show little enhancement in the presence of GABA. This modulation in BDZ receptor affinity is commonly referred to as the "GABA shift." The GABA shift is usually expressed as a ratio of the IC_{50} values (i. e., the concentration of compound required to inhibit binding of the antagonist, [^{3}H]Ro 15-1788 by 50%) in the presence and absence of GABA. BDZ receptor agonists will generate a ratio significantly less than one, while the ratio for BDZ antagonists will not differ significantly from unity.

[^{35}S]TBPS (t-butylbicyclophosphorothionate) binding is a pharmacological tool available to measure chloride interactions. Skolnick et al., (1986) reported that [^{35}S]TBPS binds to sites closely associated with the GABA-gated chloride channel. Due to the high affinity and the relatively low nonspecific binding of [^{35}S]TPBS, this compound is useful in characterizing the interrelationship of the components in the supramolecular complex. It is hypothesized, based on the effects of anions, that [^{35}S]TBPS binding is related to the permeability of the GABA gated chloride ion channels. The affinity of the radioligand is a direct measure of the permeability of these channels while the number of [^{35}S]TBPS binding sites is directly proportional to the number of GABA-gated chloride ion channels in the "open" position. Thus, [^{35}S]TBPS binding can be used as an effective probe for investigating the properties of the chloride ion channel

and the effects of both GABA and BDZ binding on this channel within the supramolecular complex.

GENETIC EFFECTS.

Strain differences among test animals present a major element of confound in extrapolating results across species. Attempts to compare human clinical states with models of animal responding are equally affected. Nonetheless, it is necessary to investigate perturbations in and across strains in order to profile possible genetic differentiation in receptor pharmacology. File and Greenblatt (1985) contrasted the ability of diazepam to block the convulsant effects of pentylenetrazol (metrazol) in four separate mice strains. Two of the strains were inbred (C3H/HE, NIH) and two were outbred strains (Tuck no1, CFLP). Pilot studies produced no significant differences in the dose of pentylenetrazol necessary to induced seizures in 100% of the mice, regardless of strain. They did, however, generate significant differences across strain in the potentiation of anticonflict activity produced by diazepam. At six hours post diazepam treatment, significant strain variability was evident. 75% protection of the C3/HE strain was sharply contrasted against less than 12.5% protection in either of the two outbred strains tested. In an attempt to explain these differences, percent receptor occupation was measured in each of the four strains. Only one outbred strain differed significantly on this measure. The investigation of which subpopulations of receptors were active in addition to total numbers of occupied receptors would have been warrented.

Finally, it is equally important to profile any differences which may exist in BDZ and chloride ionophore coupling in order to identify possible strain differences. It is useful to note that all four strains, investigated by File and Greenblatt, required higher percent receptor occupancy for 50% protection than that described in the literature (Braestrup et al., 1982). This apparent deviation may account partially for the prolonged anticonflict effects noted in these mice. Behavioral anti-conflict effects were reported as long as six hours post treatment, while the average protection time reported in the literature is between 30 and 60 minutes.

Acute tolerance toward the BDZ's may explain these results as further study is warranted.

Gallaher (Gallaher et al., 1987) has developed two genetic lines of mice, through inbreeding based on their responsiveness to the sedative effects of diazepam (Valium). One line displayed marked tolerance to Valium and was designated as resistant (DR), while the other line was markedly sensitive to these sedative effects and was labeled diazepam sensitive (DS). Gallaher profiled the sedative effects of various BDZ's in both strains of mice. The authors operationally define sensitivity as a measure of the animal's ability to remain on the rotarod (a slowly rotating dowel: 10rpm) following the administration of 20 mg/kg diazepam. The initial test group was rated on a scale from 0 to 300 based on the "duration of stay" on the rotarod. Animals remaining on the rotarod for thirty minutes were assigned a score of 0 while those animals still impaired 300 minutes following diazepam administration were assigned a score of 300. Gallaher randomly selected, regardless of test score, 10 males and 10 females for purposes of breeding a control line. The drug resistant line was bred from mice assigned scores of 0 while the drug sensitive line was bred from those mice assigned the highest scores. Mating between siblings was avoided whenever possible. Each successive generation was tested against the rotarod as well. The first generation DS/DR sample differed at $p \leq .05$ on rotarod performance. Thereafter, the second and future generations differed at $p \leq .001$. These statistical differences reflect real behavioral differences between these two lines of genetically selected mice

Biochemical analysis of the two strains did not yield significant differential effects. One possibility might be that Gallaher may have selected for a physiological impairment in the ability of the DR lines to absorb and transport the BDZ to the appropriate receptor sites. In order to address this issue, Gallaher tested for divergence in the bioavailability of diazepam. He randomly selected 10 DR and 10 DS Male mice, administered 20 mg/kg diazepam and sacrificed the animals thirty minutes later.

The brains were assayed for differential BDZ levels. No significant difference was reported in the DR line (1297 ± 300 ng/g) and the DS line (1318 ± 277 ng/g). Gallaher has suggested that the biochemical mechanism responsible for the differential behavioral effects across strain, is not caused by absorption, metabolic or excretory rates, but rather by perturbations in the CNS which alter drug efficacy. These alterations may be directly manifested through changes in the coupling mechanisms between the BDZ and GABA related receptor sites. Endogenous substances or ligands responsive to the BDZ complex have been postulated to account for the differential behavioral response across these two lines. To date, however, no endogenous ligands have been identified for the BDZ's which function in a manner similar to the endogenous opiates and the endorphin system.

It is apparent from the above discussions that the BDZ and GABA receptor and chloride ionophore sites are complex and involve mutual interaction. Some of these interactions are direct and are guided by intricate coupling pathways, while some might depend on the cell environment in which the supramolecular complex finds itself. Further clarification of the BDZ, GABA and chloride ionophore complex will follow from future study. The information rendered will expand our conceptual understanding of the pharmacological mechanism driving the anxiolytic response. To date, the BDZ model explains clearly and precisely the vast compendium of data which has been generated on the behavioral and biochemical mechanisms involved in the mediation of anxiety.

Since the drug sensitive and drug resistant mice were developed through inbreeding, based on their sedative effects to diazepam, a genetic difference in their response to the BDZ's would be suggested. These differences may be reflected through BDZ receptor processes, genetically determined through DNA coding. No measure of the anti-anxiety effects of the BDZ's on the DS and DR mice have been investigated. Therefore, both behavioral and biochemical profiles of the DS and DR mice will be performed in order to assess their genetically selected response to the anxiolytic effects of the BDZ's.

BEHAVIORAL TRAINING AND TESTING METHODOLOGY

SUBJECTS. The mice used in this study were derived from a HS/IBg heterogeneous strain. They were selectively bred over multiple generations for their enhanced response to the sedative effects of a single dose of diazepam. The sedative effects were measured by the animals performance on the rotarod test for neurological impairment (Dunham and Miya, 1957). Mice were scored according to their ability to balance for 30 sec on a slowly rotating dowel (10 rpm) suspended 46 cm above a bed of sawdust (Gallaher, 1982). Litters from the parents of the ninth generation were used for these behavioral studies. Twelve drug sensitive and twelve drug resistant mice were randomly selected with a mean weight of 28 ± 2.5 grams. Twelve control mice were similarly selected from the control line of HS/IBg mice with a mean weight of 34 ± 3.0 grams. Mice were housed in a small rodent room, temperature maintained at 22°C ± 2°C. Lights were on between 0600 and 1800 hours. The animals, after arriving at the facility, were given a minimum of fourteen days in which to acclimate before any training was implemented. During this time the animals had free access to Purina laboratory rat chow and water. Weights were recorded on a daily basis throughout behavioral training and testing.

MATERIALS. [^{3}H]flunitrazepam (76.9 Ci/mmol) was purchased form New England Nuclear (Boston, MA.). Sodium chloride (0.9%) was purchased from Abbott laboratories, North Chicago, Illinois. Absolute ethanol was purchased from USI Chemical Co., Tuscola, Illinois.

APPARATUS. Animals were tested in a standard rodent operant box modified for use with mice (Appendix 2). The

plexiglass compartment measured 16.0 cm square. The floor consisted of 23 stainless steel bars spaced 0.50 cm apart. A small circular hole 1.0 cm wide was cut on one side of the box through which a water bottle spout was inserted. The hole was placed 7.5 cm from the sides of the panel. A two pole shock generator was connected to the water spout and grid floor. The light source consisted of a twelve foot-candle transparent light bulb, suspended 2.0 cm from the top of the operant chamber. The apparatus was enclosed in a sound insulated, fan ventilated chest (89 x 38 x 38 cm) with a plexiglass window located directly in front of the mouse operant chamber. The stimulus light, water tube, shock generator and cumulative recorder were all controlled through solid state operant control equipment furnished by Gerbrands. A Gerbrands cumulative recorder measured the number of shocks delivered during the punished interval, the number of licks taken during the punished interval, the number of licks taken during the free period and the total number of licks taken throughout the testing period. Continous contact with the water spout was automatically recorded at a rate of 7 licks per sec. In addition, cumulative recordings were taken during all testing conditions. Individual records were kept of these data, on a daily basis, across all training and testing sessions.

PROCEDURE. Certain physical criteria were established in order to formalize the training protocol. The subject's weight was monitored daily. Deprivation conditions were temporarily suspended if a subjects weight decreased to 80% of the free feeding weight. Fifteen minutes of additional water was made available daily in the home cage, in addition to the water the mice received during the test. The shock intensity was monitored and titrated to the individual mouse response data. In order to optimize suppression during punished responding, shock levels were slowly adjusted from .1 mA to a maximum of .3 mA, based on the ratio of punished responses to free licking. Shock levels were increased until the punished responses, on average, did not exceed 10%-20% of the total responses made during the test

session. Shock levels were evaluated on a daily basis and adjustments made in order to counteract habituating effects and readjust to baseline.

Training: Control Mice
Phase 1: Acquisition of response

The control mice were individually trained, over an eight week period, in order to investigate the effects of modifications in variables such as reinforcement-punishment contingencies, shock parameters and deprivation conditions prior to profiling the DS and DR mice. The control mice were water deprived 48 hr prior to their first exposure to the test environment. The animals were trained on 5 consecutive days each week. Water was available in the home cage for 30 min on each of the two remaining days. During the first week of training, the mice were placed in the test chamber for a 15 min adaptation period, during which the animals had free access to the drinking tube. The external stimulus light was off during the full 15 min and no punishment was delivered. The initial training sessions lasted one week and facilitated the acquisition of a stable baseline response.

Phase II: Conflict Training

During the second week, the 15 min test period was sub-divided into one 2 min and one 13 min interval. During the first two minutes, the light source was on and shock (.1 mA) was delivered following each tenth lick (FR=10). Shock was subsequently delivered on this schedule throughout training and testing. A timer was activated at the termination of the first shock and marked the two minute shock period, after which the light source was automatically extinguished. This interval was followed in succession by 13 min of free drinking (no shock). The shock interval was increased to 4 min during the third week with reciprocal changes in the free period. The shock intensity was also adjusted from .1 mA to .2 mA. Cumulative recordings of

responses were taken for each individual training period. These records were reviewed daily and the shock levels adjusted accordingly, in order to maximize inhibition of punished responding without extinguishing response behavior indiscriminately. By the fourth week of training, each mouse was exposed to 5 min of punished responding followed by 10 min of free licking. The shock level for some animals was increased to .3 mA depending on their individual suppression ratios.

After five weeks of training (to acquire stable suppression of punished responding and consistent baseline responding during the free interval) the shock interval was increased to 7.5 min in order to facilitate simple comparison of punished and unpunished responding. Accordingly, the unpunished interval was decreased to 7.5 min. Two weeks of training followed during which time suppression and unpunished responding levels readjusted to baseline. Drug profiles were implemented once the suppression ratios reached 10-20% of total responding.

Phase III: Drug Testing

Several doses of chlordiazepoxide were prepared as solutions in physiological saline. The doses included: 2.5, 5.0, 7.5, 10.0, 20.0, 30 mg/kg. The injection volume in all cases was 0.1 ml per 10 grams body weight and the animals were tested 30 min after injection. A vehicle control (saline) was also tested. In an attempt to minimize any carry-over effects, only one dose was administered each week. The dose schedules were randomized and each dose was tested in triplicate per subject. The animals were tested under the same experimental conditions, without drug, for the remaining four days of each week.

Each subject was placed into the test chamber 30 min after intraperitoneal injection. A switch activated a timer, which marked two 7.5 min intervals. The timer also controlled the stimulus light source. The mouse was delivered mouth shock on an FR=10 schedule during the first 7.5 min. The timer automatically turned the light source off at the end of the first 7.5

min period, signaling the end of the punished interval and the beginning of the free (unpunished) response segment. The animal was free to lick for the remaining 7.5 min, after which they were promptly removed from the test environment.

Training: Drug Sensitive and Drug Resistant mice
Phase I: Acquisition of response

The drug sensitive (DS) and drug resistant (DR) mice were trained to criteria, according to the parameters set forth in the protocol established for the control mice. The DS and DR mice were deprived of water for 48 hr prior to the innitial exposure to the testing environment. The animals were adapted to the test chamber during which time they had free access to the drinking bottle for the entire 12 min period. The testing interval was shortened by 3 min in order to increase the number of animals tested per day. The external stimulus light source was not on during this time. This procedure was repeated for 5 days or until the acquisition of baseline response was met. In order to compensate for variations in intake volume, each mouse was supplemented with an additional 15 min of water in their home cage immediately following the training period,.

Phase II: Conflict Training

Low intensity shock was introduced after the successful acquisition of the response baseline. Shock was administered for a fixed interval of time at the onset of the trial. The subject was placed into the test chamber and the light source (external stimulus cue) activated by a timing device. The subject was permitted to find the drinking tube and complete 10 licks before shock was administered on an FR=10 schedule. The duration of the shock was 2 sec and controlled by withdrawal from the tube. The shock interval initially lasted 1 min and was increased according to the animals suppression ratio. Shock intensity was adjusted accordingly as well. The goal of the training was to

increase the shock interval to 6 min while maintaining a specific suppression ratio. The suppression ratio was operationally defined as the percent of punished responding as compared to total responses taken during the 12 min test interval. Baseline suppression criteria were set not to exceed 20% of the total responses made during the 12 min session. The shock was applied at the water tube and the intensity varied from 0.1 mA - 0.3 mA. The subject was trained to inhibit responding during the cued 6 min shock interval and then allowed to drink freely for the remaining 6 min. The number of shocks received, number of licks in punished period, number of licks in non shocked period and total number of licks were recorded for each session, as were cumulative recordings for the session.

Phase III: Drug Testing

Thirty minutes after intraperitoneal injection, each subject was placed in the apparatus. Drug doses were prepared as solutions in physiological saline. Each animal in the control, drug sensitive and drug resistant groups was randomly administered one of 6 doses of chlordiazepoxide and a vehicle control (saline). Each dose was administered three times to each animal throughout the course of the experiment. Due to the attenuated responsiveness in the drug resistant mice, an additional dose was added to the drug profile for this group. The DS mice were administered 2.5, 5.0, 7.5, 10.0, 20.0 and 30.0 mg/kg chlordiazepoxide while the DR mice received 2.5, 5.0, 7.5, 10.0, 20.0, 30.0 and 40.0 mg/kg chlordiazepoxide. A switch activated a timing device which marked two 6 min intervals. The switch automatically activated the light source at the beginning of the first 6 min (punished) period and extinguished the stimulus at the beginning of the second 6 min (unpunished) interval. The shock was delivered on an FR=10 schedule with a duration of 2 sec. The shock intensities were predetermined from data collected during the training schedules for each subject. Once the external cue was withdrawn, the animal was free to drink for the remaining 6 min. At the end of each 12 min test session, the animal was removed from the test apparatus and returned to the home cage, where access to water was allowed for an additional 15 min.

Potentiation of Ethanol by Chlordiazepoxide

SUBJECTS. The mice used in this study were drawn from the ninth generation HS/IBg strain. A group of naive mice from the ninth generation HS/IBg strain were randomly selected. Twelve drug resistant, 12 drug sensitive and 8 controls were profiled for the potentiating effects of chlordiazepoxide on ethanol. In addition, the mice trained on conflict were also profiled for ethanol potentiation 4 wks after conflict testing had ended.

PROCEDURE. During each testing period, the DR, DS and control mice, randomly selected from the ninth generation HS/IBg strain, were pretreated with a vehicle control (saline) or one of the following doses of chlordiazepoxide: 2.5, 5.0, 10.0 or 20.0 mg/kg. The injection volume in all cases was 0.1 ml per 10 grams body weight. Thirty minutes later, 3 g/kg of absolute ethanol was administered via intraperitoneal injection. Onset and end of narcosis were recorded for each animal. Narcosis was operationally defined as the loss of righting reflex for the minimum duration of one minute. Narcosis was terminated when the animal spontaneously righted itself and would not remain on its back when placed there by the experimenter. The tests were conducted at one week intervals with each test period lasting approximately 3 hr. The experimental schedule initially tested ethanol in the presence of the vehicle control (saline). The four doses of chlordiazepoxide were randomly administered over the course of twelve weeks. Only one dose of chlordiazepoxide was tested in any given session. Data was recorded on the weight of the animal, the time of ethanol administration, the time of loss of righting and the time the animal regained the righting reflex.

The drug sensitive, drug resistant and control mice which had been previously trained, were also profiled on this measure of potentiation. These mice, however were pretreated with the vehicle control and 10 mg/kg chlordiazepoxide prior to the

administration of 3 g/kg ethanol. Data was recorded on the weight of the animal, the time of ethanol administration, the time of loss of righting and the time the animal regained the righting reflex.

BIOCHEMICAL TESTING METHODOLOGY

MATERIALS. An Ultra Turrax Tissumizer (Teckmar Co. Cincinatti, Ohio) and Beckman J2-21M Induction drive centrifuge were used to prepare homogenates. The assay tubes were filtered using a Brandell M-24R cell harvester. A Beckman LS 2800 Liquid Scintillation counter was used to measure the radioactive ligand bound to BDZ receptors.

Preparation of mouse brain crude-P2 membranes for [^{3}H] Flunitrazepam binding.

Drug sensitive and drug resistant mice, derived from the ninth generation HS/IBg strain, and control albino ICR mice (Royalhart) were sacrificed by decapitation and the brains quickly removed and placed on ice. The cerebral cortex, cerebellum and hippocampus were dissected, weighed, and frozen separately at -20°C. No tissue from the hippocampus was available from the control mice. Tissue samples were pooled in the hippocampus and cerebellum sample, within populations, to produce the required protein concentrations for [^{3}H]flunitrazepam binding studies. The tissue was thawed two days prior to the binding assay. The tissue from each brain region was suspended in 15 volumes (1 g/15 ml) of ice cold 0.32 M sucrose -10 mM HEPES buffer (pH 7.4) and homogenized using ten strokes with a Thomas glass-teflon tissue grinder (50 rpm). Homogenates were centrifuged for 10 min at 1000 x g at 4°C. The pellets were discarded and the supernatant solutions re-centrifuged at 48,000 x g for 20 min at 4°C. The resulting pellets were resuspended in 15 volumes of ice cold distilled water and homogenized using a Teckmar Tissumizer, set at 60 rpm for 15 sec. The homogenate was then centrifuged at 48,000 x g for 20 min. The resulting pellets were centrifuged at 48,000 x g for 20 min and washed twice in 15 volumes of 40.5 mM Na / 9.5 mM K phosphate buffer (pH 7.4) at 4°C. The final

pellets were resuspended in 15 volumes of Na/K PO4 buffer and stored in 3 ml aliquots at -20°C. In order to optimize the removal of endogenous GABA, the aliquots were frozen for a minimum of 18 hr prior to thawing.

On the day of the binding assay, the aliquots were thawed and centrifuged at 48,000 x g for 20 min. The resulting pellets were resuspended in 15 volumes of Na/K PO4 buffer and recentrifuged at 48,000 x g for 20 min. The pellets were resuspended and centrifuged twice. The final pellets were resupended in the appropriate volume of Na/K PO4 buffer to give a protein concentration of 1 mg/ml. Protein concentrations were determined by the method of Lowry et al., (1951).

[^{3}H]Flunitrazepam Binding Assay ± GABA

Triplicate aliquots (100 µl) of tissue were incubated with 50 µl of [^{3}H]flunitrazepam (.323, .696, 1.50, 3.23, 6.96 and 15 mM final concentration), 50 µl of 50 mM Na/K phosphate buffer (pH 7.5) and with or without 10 µm GABA (final concentration) at 4°C for 75 min. BDZ binding studies were performed as described by Garrett and Tabakoff (1985). The reaction was terminated by rapid filtration under vacuum (15 mm/Hg) through Whatman GF/C filters using a 24 well Brandell cell harvester. The tubes were washed twice with 5 ml ice cold 50 mM Na/K phosphate buffer. The Whatman GF/C filters were placed into glass scintillation vials together with 5 ml of (Beckman Bio solv) scintillation cocktail. The radioactivity on the filters was determined by standard scintillation counting techniques using a Beckman Scintillation counter (LS 2800) with an efficiency rating of .54. Non-specific binding was determined by including 1 µM clonazepam in a corresponding set of samples and represented approximately 5% of total binding. Specific binding was calculated by subtracting non-specific binding from total binding counts. Protein was determined by the method of Lowry et al., (1951) using bovine serum albumin as the standard. All reagents and

drugs were diluted in 50 mM Na/K phosphate (pH 7.4) and the total volume of the assay was 250 µl.

Preparation of mouse cortex for [^{35}S]TBPS binding

Cortical membranes for [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) binding were prepared using modifications of the method of Lildjquist and Tabakoff (1985). Drug sensitive and drug resistant mice, from the ninth generation HS/IBg strain, were sacrificed by decapitation. The brains were quickly removed and placed on ice. Cerebral cortex was dissected and homogenized in 15 volumes of cold 0.32 M sucrose-10 mM HEPES, pH 7.4 at 4° C, using a Thomas glass-Teflon tissue grinder. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was carefully decanted and centrifuged at 48,000 x g for 20 min. The resulting pellet (P2) was homogenized, for 15 seconds, in cold distilled water using a Teckmar Tissumizer set at 60 rpm. The suspension was centrifuged at 48,000 x g for 20 min and the pellet washed twice with 50 mM Tris-citrate buffer, pH 7.4 at 4°C. The resulting pellet was resuspended in 15 volumes of Tris-citrate buffer and frozen at - 20°C for 24 hr in order to facilitate the removal of endogenous GABA.

One day later the pellets were thawed and centrifuged at 48,000 x g for 20 min. The pellets were washed twice with 15 volumes of Tris-citrate buffer. The resultant membrane solutions were stored in 2 ml aliquots and frozen in liquid nitrogen. On the day of the binding assay, the aliquots were thawed, washed twice and resuspended in a sufficient volume of 50 mM Tris-citrate, pH 7.4 at 20°C, to render a final protein concentration of 1.0 mg/ml.

[^{35}S]TBPS Binding Assay

[^{35}S]TBPS binding was assayed by a modification of the method of Ramanjaneyulu and Ticku (1983). Triplicate aliquots of cortical tissue (100 µl) were incubated with 2 nM [^{35}S]TBPS (50 µl), 200 mM NaCL (50 µl) and .00316-100.0 µM (final concentration) diazepam (50 µl) for 60 min at room temperature. The mixtures were then filtered under vacuum (15 mm Hg) and washed twice with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) using a Brandell cell harvester. The filters were placed into glass scintillation vials together with 5 ml of (Bio-solv Beckman) scintillation cocktail. The vials were shaken vigorously and the radioactivity on the filters measured using conventional scintillation counting techniques. Non-specific binding was determined by including 100 µM picrotoxin in a parallel set of samples. Specific binding was calculated by subtracting non-specific binding from total binding counts. All reagents and drugs were diluted in 50 mM Tris-citrate, and the total volume of the assay was 250 µl. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

BEHAVIORAL RESULTS:

The mean number of safe responses made by the DR, DS and control mice is presented in Table 1. In order to successfully compare dose response functions across strain, it was necessary to first train the acquisition of the response as well as stable baseline responding, over time, at each of the ascending shock intervals. The mean and standard errors presented in Table 1, for the drug resistant and drug sensitive mice show strong stability across the shock periods. The standard error for each of the mean number of responses is extremely low, suggesting low variability in the rate of intersubject responding within strain. The mean number of safe responses for the control sample is more variable and reflects changes implemented in the development of the conflict procedure. The standard errors are slighly higher, as well, reflecting greater variation among control subjects in their rates of responding. Mean shock levels did not significantly different across strain. During acquisition, shock levels were titrated between .1 and .2 mA in the control, DR and DS mice. The strain average did not differ by more than .02 mA. During the presentation of shock, the shock level was titrated from .2 to .3 mA and the mean shock levels did not differ by more than .01 mA.

Control Mice

Phase I: Acquisition of Baseline

A representative sample of the individual daily responses made during the acquisition of the non-punished baseline response is presented in the first panel of Figure 1A. These cumulative recordings were taken over a 15 min test period, during which punished responding and free licking was measured.

Table 1

Behavioral Training
Mean number of Safe Responses (± SEM)

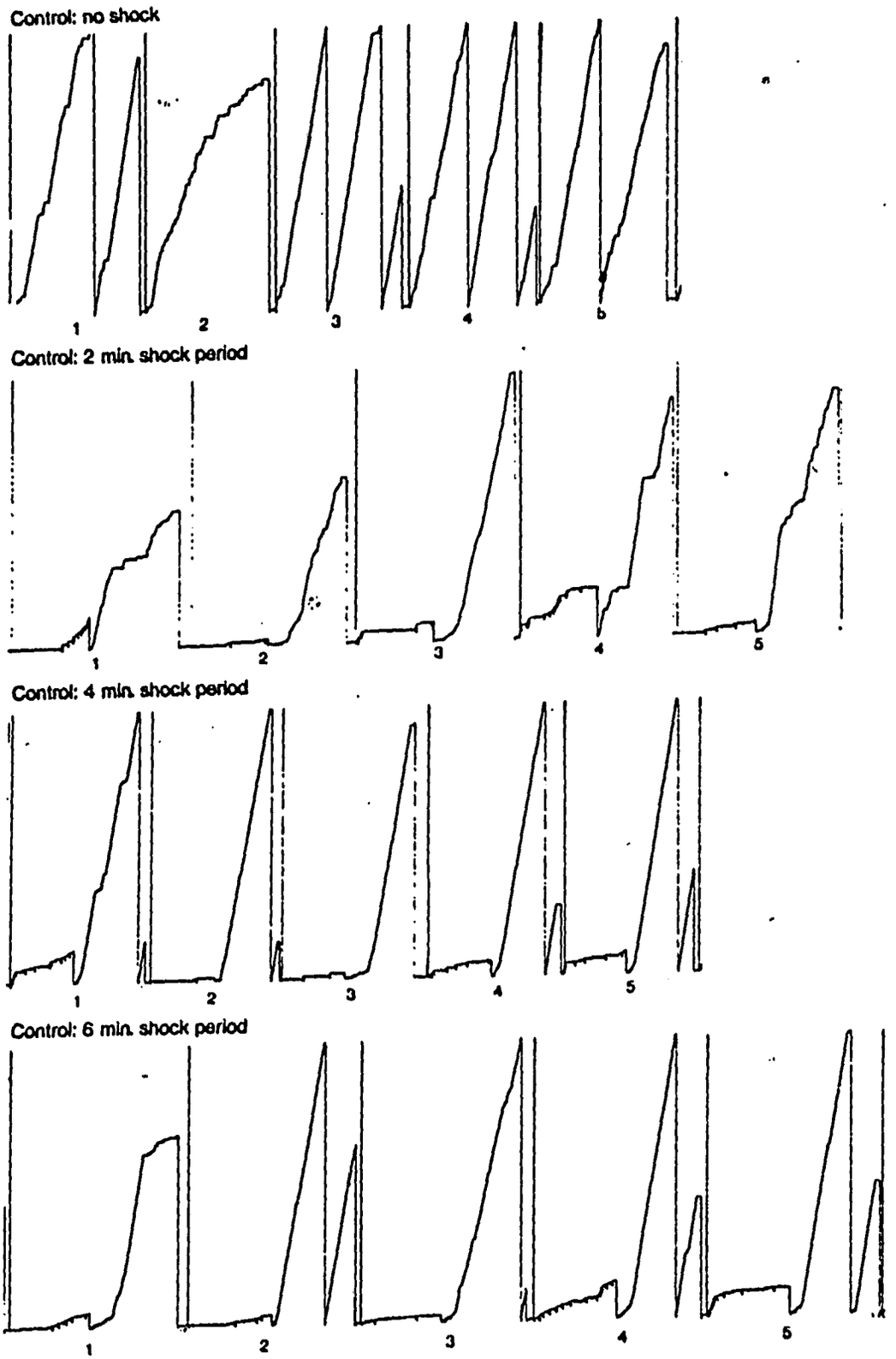
	Acquisition (no shock)	2 min shock interval	4 min shock interval	6 min shock interval
RESISTANT	1198.6 (±70.3)	1369.1 (±39.1)	1342.8 (±44.8)	1248.7 (±45.2)
SENSITIVE	1271.2 (±36.1)	1292.2 (±38.2)	1287.5 (±31.8)	1166.1 (±39.1)
CONTROL	795.9 (±60.2)	1331.1 (±49.4)	1259.2 (±43.4)	1082.6 (±60.1)

Figure 1A

Control Mice:
Behavioral Training Records

Cumulative behavioral training records for the acquisition of response in the absence of aversive stimuli and subsequent suppression in the presence of shock. Pen deflections correspond to delivery of shock. Subject code numbers are provided under each corresponding record.

Figure 1A


Control: no shock
1
2
3
4
5
Control: 2 min. shock period
1
2
3
4
5
Control: 4 min. shock period
1
2
3
4
5
Control: 6 min. shock period
1
2
3
4
5

The slope of the cumulative recordings represents the rate at which the animal licked the water tube; the steeper the slope, the higher of the rate of licking. These recordings demonstrate the successful acquisition of the licking response and the robust and consistent nature of the trained response over time. The similarity across individual response rates is also high and can be illustrated by the relative consistency in the height and slope of each of the consecutive recordings.

Phase II: Conflict Training

Shock intervals were introduced following the acquisition of baseline responding. Figure 1A presents representative weekly profiles for five animals throughout conflict training. The second panel in Figure 1A illustrates the acquisition of the suppression levels. The first segment of the recording represents the shock period. Each pen deflection corresponds with the delivery of a shock (FR=10 licks). Close inspection of the records will show that the responses made during the shock interval, on average, do not exceed 20% of the responses made during the free interval. This corresponds with a maximal suppression ratio of 10 shock licks to every 50 free licks. The criteria was initially set to attain a suppression ratio of 20% or less. The recordings demonstrate a suppression ratio even higher in most cases. Figure 1A illustrates the training of suppressed responding during the cued presentation of shock and the potentiation of responses during the free period. These records also illustrate intersubject uniformity and consistency in response rates throughout the six week training period.

Drug Sensitive and Drug Resistant mice
Phase I: Acquisition of response

The training protocols outlined above for the control mice were followed for both the DS and DR mice. Acquisition of baseline responding was accomplished in 3 weeks with less

subject variability. This can be confirmed by inspection of Figures 1B and 1C which correspond to the cumulative records for the training of the DS and DR mice respectively. The height and slope of the cumulative recordings is significantly greater for the DS and DR mice when compared to the controls. In addition, the DS and DR mice show remarkable intersubject concurrence in response rates. Based on the data presented in Figure 1B and 1C, the individual cumulative records for acquisition are almost superimposable and remain so throughout training. Intersubject baseline responding across the DS and DR strains is also highly consistent.

Phase II: Conflict Training

Similar methodological procedures were followed for the DS and DR mice as elaborated in the previous discussion of the conflict training for the control mice. Panels 2-4 of Figure 1B and 1C present representative weekly cumulative records for the introduction and training of suppression rates in responding during the shock interval. Three weeks of training were necessary in order to establish baseline suppression levels during the six minute shock interval. Both figures clearly illustrate the relative increase in response rate during the free period. The slope and heights for the free periods are significantly greater than for the shocked intervals. The training methodology, as reflected by these figures, developed a robust, consistent effect in responding over time which generated comparative baseline levels in shocked response suppression for each of the two genetic lines.

Figure 1B

Drug Sensitive Mice:
Behavioral Training Records

Cumulative behavioral training records for the acquisition of response in the absence of aversive stimuli and subsequent suppression in the presence of shock. Pen deflections correspond to delivery of shock. Subject code numbers are provided under each corresponding record.

Figure 1B

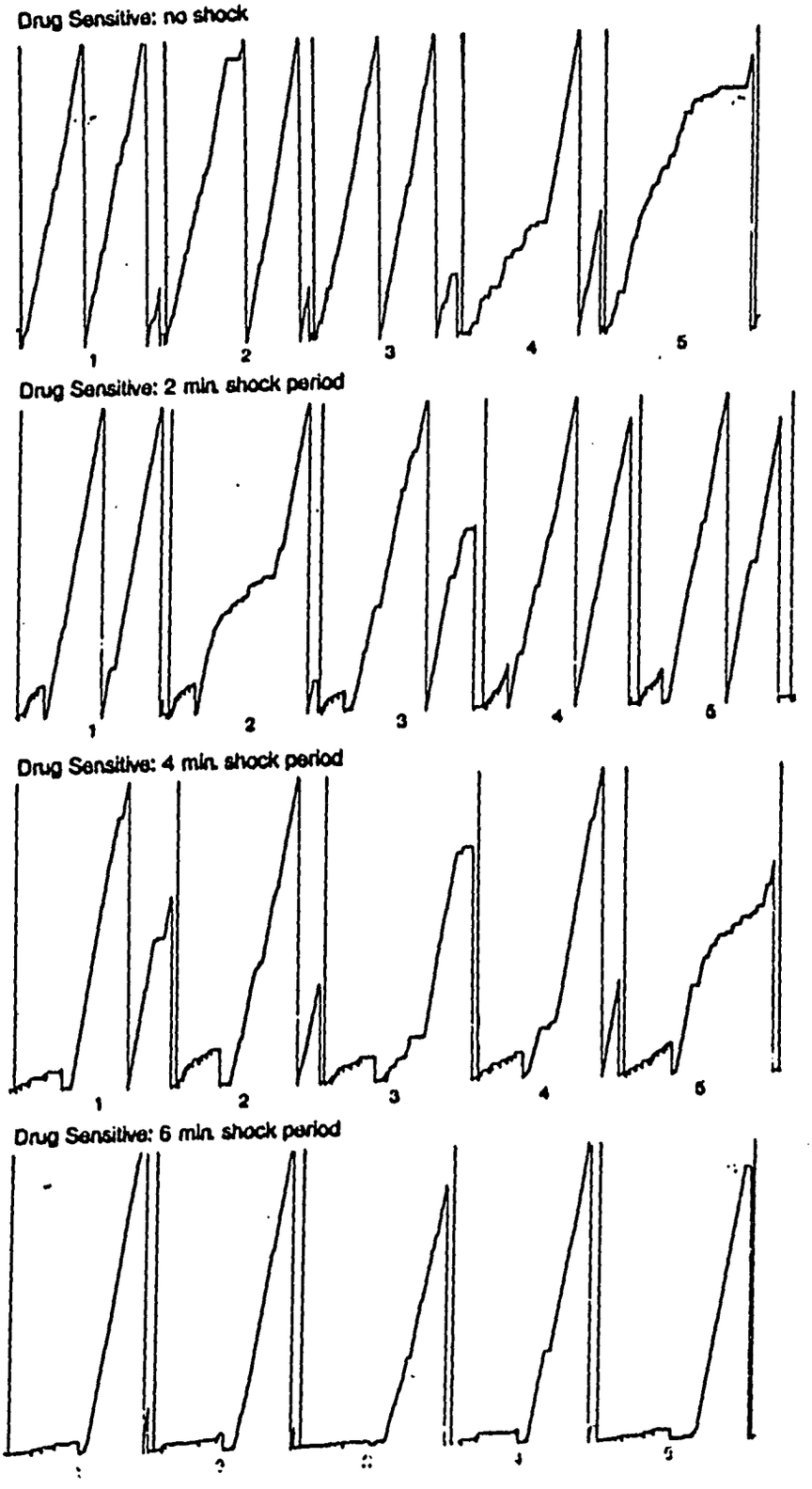
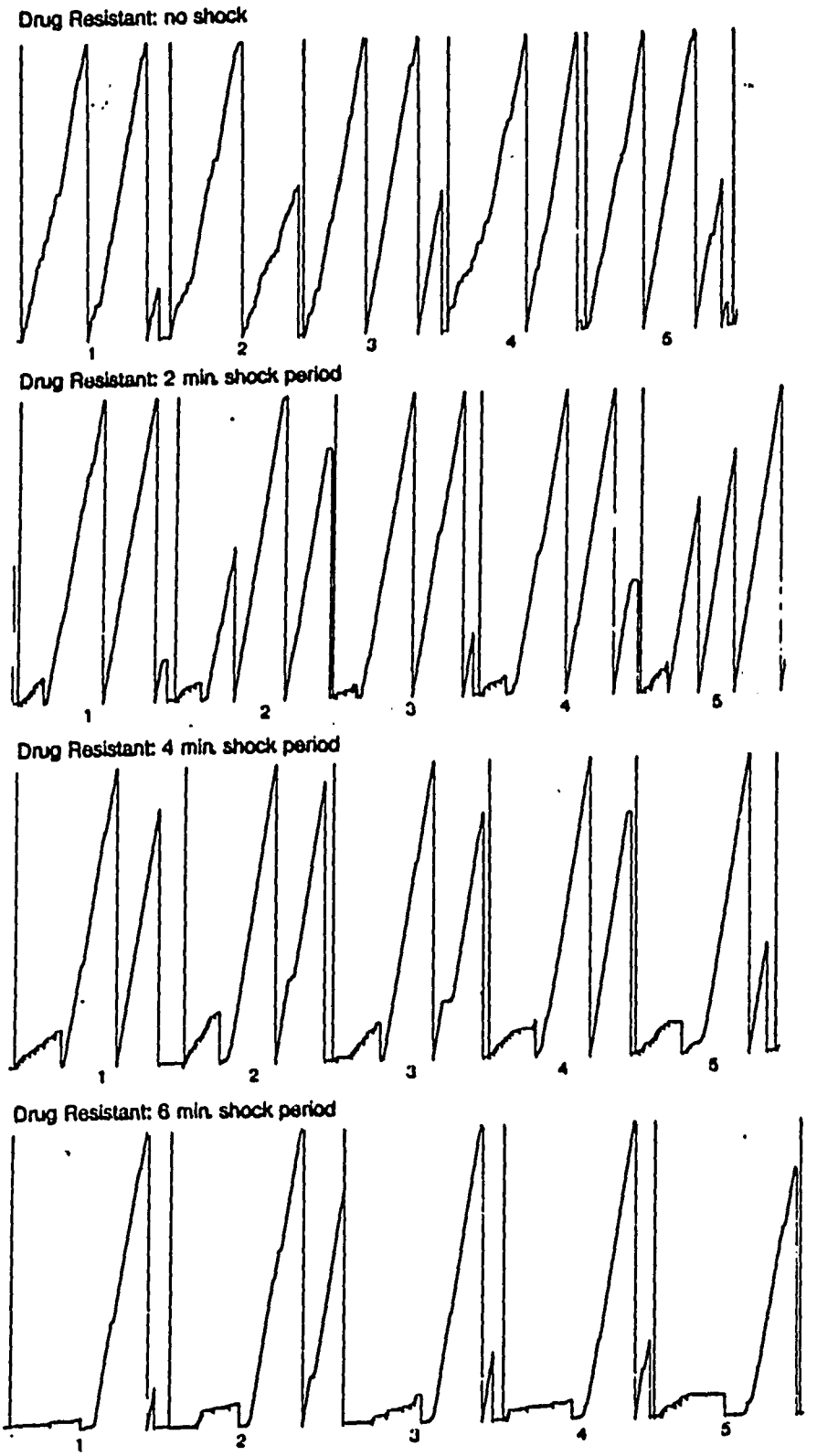

Drug Sensitive: no shock
1
2
3
4
5
Drug Sensitive: 2 min. shock period
1
2
3
4
5
Drug Sensitive: 4 min. shock period
1
2
3
4
5
Drug Sensitive: 6 min. shock period

Figure 1C

Drug Resistant Mice:
Behavioral Training Records

Cumulative behavioral training records for the acquisition of response in the absence of aversive stimuli and subsequent suppression in the presence of shock. Pen deflections correspond to delivery of shock. Subject code numbers are provided under each corresponding record.

Figure 1C


Drug Resistant: no shock
1
2
3
4
5
Drug Resistant: 2 min. shock period
1
2
3
4
5
Drug Resistant: 4 min. shock period
1
2
3
4
5
Drug Resistant: 6 min. shock period
1
2
3
4
5

Phase III: Drug Testing

The number of shocks received was averaged across the three repetitions of each dose for each animal. Mean dose response profiles for each subject are presented in Figures 2A, 2B and 2C. The group mean drug -response functions are shown in Figure 3 for control, resistant and sensitive mice. For each group, two phases in the dose-response profile were evident. First, there was a gradual increase in the number of shocks tolerated with increasing dosage. This increase was followed by a sudden decrease in response at higher doses. Each stage was evaluated separately, given the apparent biphasic nature of the dose-response profile. To that end, the range of concentrations was split into two segments, one corresponding to the anxiolytic phase, the other to the "high dose" phase. Specifically, data in the 2.5 to 20 mg/kg range were handled by one method of analysis, while the data at 30 mg/kg and above were treated separately.

Effects in the 2.5 to 20 mg/kg dose range:

To adjust for differences among subjects at baseline, the data were expressed as a percent of baseline (Figure 4). The baseline was operationally defined as the average response of the three readings for a given animal when administered the vehicle control. A log transformation was used to linearize the observed dose-response profile over the dose range. Figure 5 indicates that log transformations of the response data were not entirely satisfactory in generating linear functions. Figure 6 shows the profiles for the mean responses when both the response and dose are expressed on the log scale. These log transformations are reasonably linear. It is evident that the dose -response curves for the control and drug sensitive groups are very similar, while the profile for the drug resistant group produces a similar slope, but is significantly shifted to the right. The relative displacement, to the right, of the dose response curves in the drug resistant animals accounts for the need for higher doses of chlordiazepoxide

Figure 2A

Control Mice:
Dose Response Profiles

Mean dose response profiles based on three replications of each dose for each animal. The graph indicates low variability across subjects and identifies 20 mg/kg chlordiazepoxide as the maximally effective anxiolytic dose.

Figure 2A

CONTROL: DOSE RESPONSE PROFILES

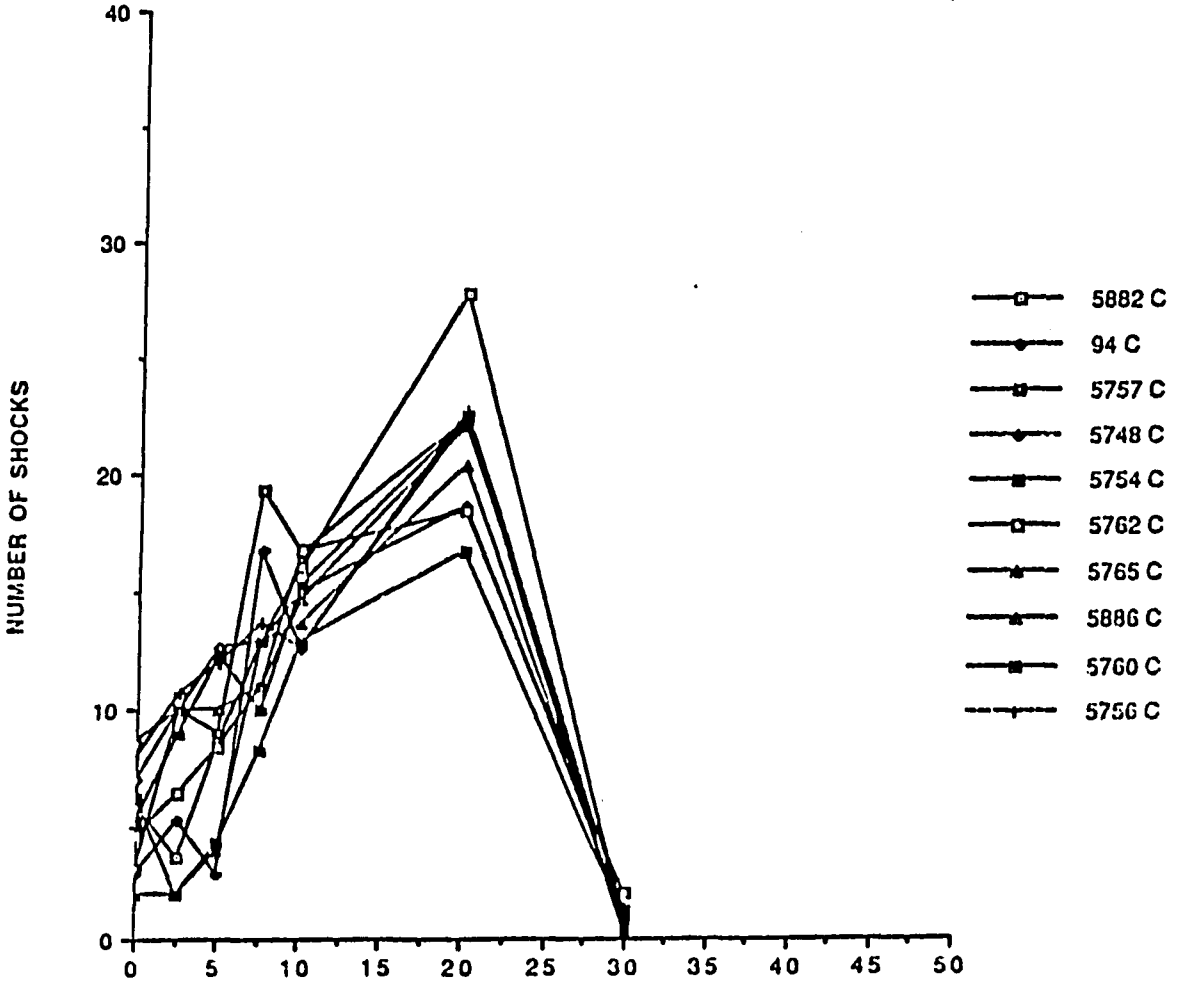


Figure 2B

Drug Resistant Mice:
Dose Response Profiles

Mean dose response profiles based on three replications of each dose for each animal. The graph indicates increased variability across subjects and identifies 30 mg/kg chlordiazepoxide as the maximally effective anxiolytic dose.

Figure 2B

DRUG RESISTANT: DOSE RESPONSE PROFILES

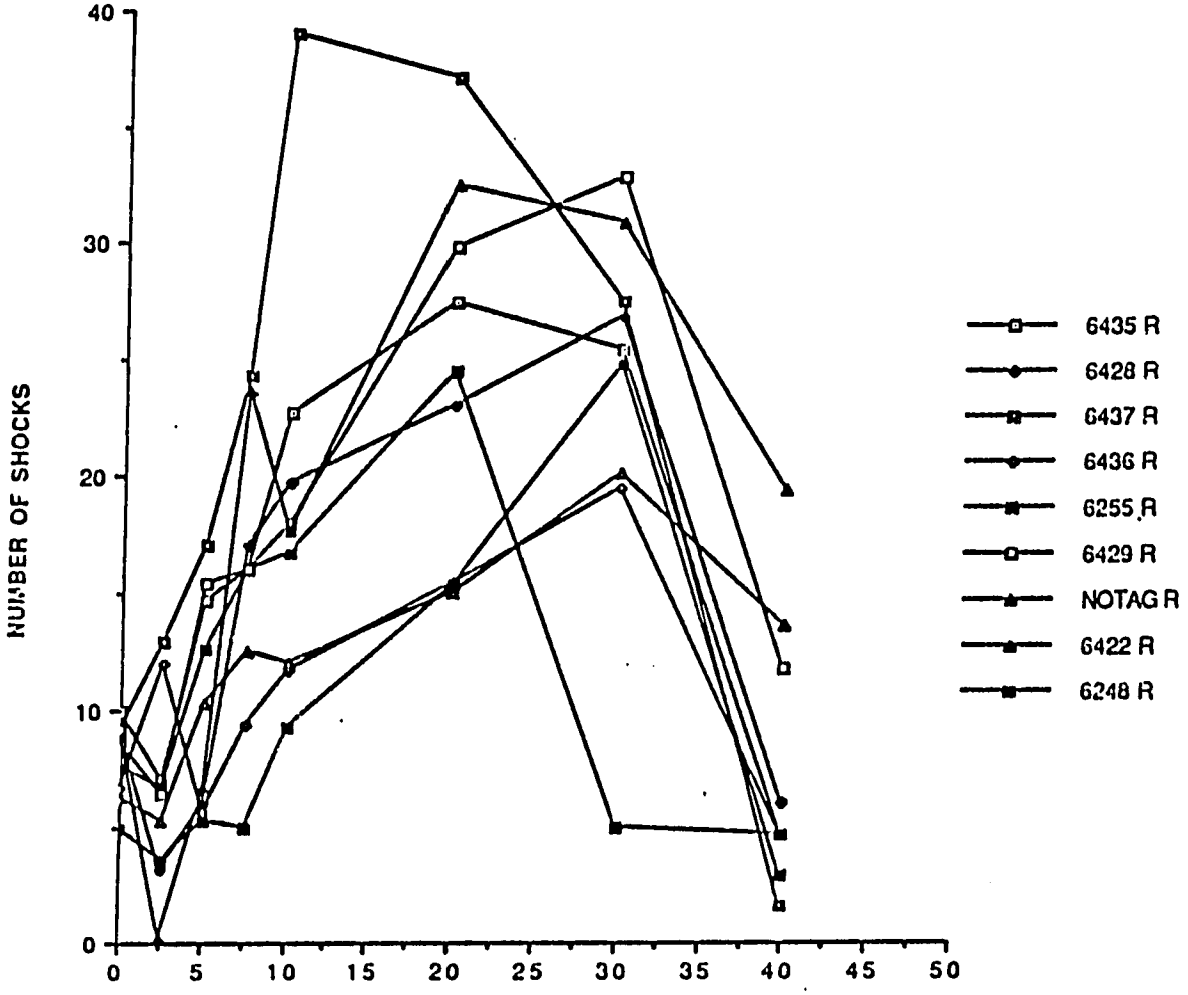


Figure 2C

Drug Sensitive Mice:
Dose Response Profiles

Mean dose response profiles based on three replications of each dose for each animal. The graph indicates low variability across subjects and identifies 20 mg/kg chlordiazepoxide as the maximally effective anxiolytic dose.

Figure 2C

DRUG SENSITIVE:DOSE RESPONSE PROFILES

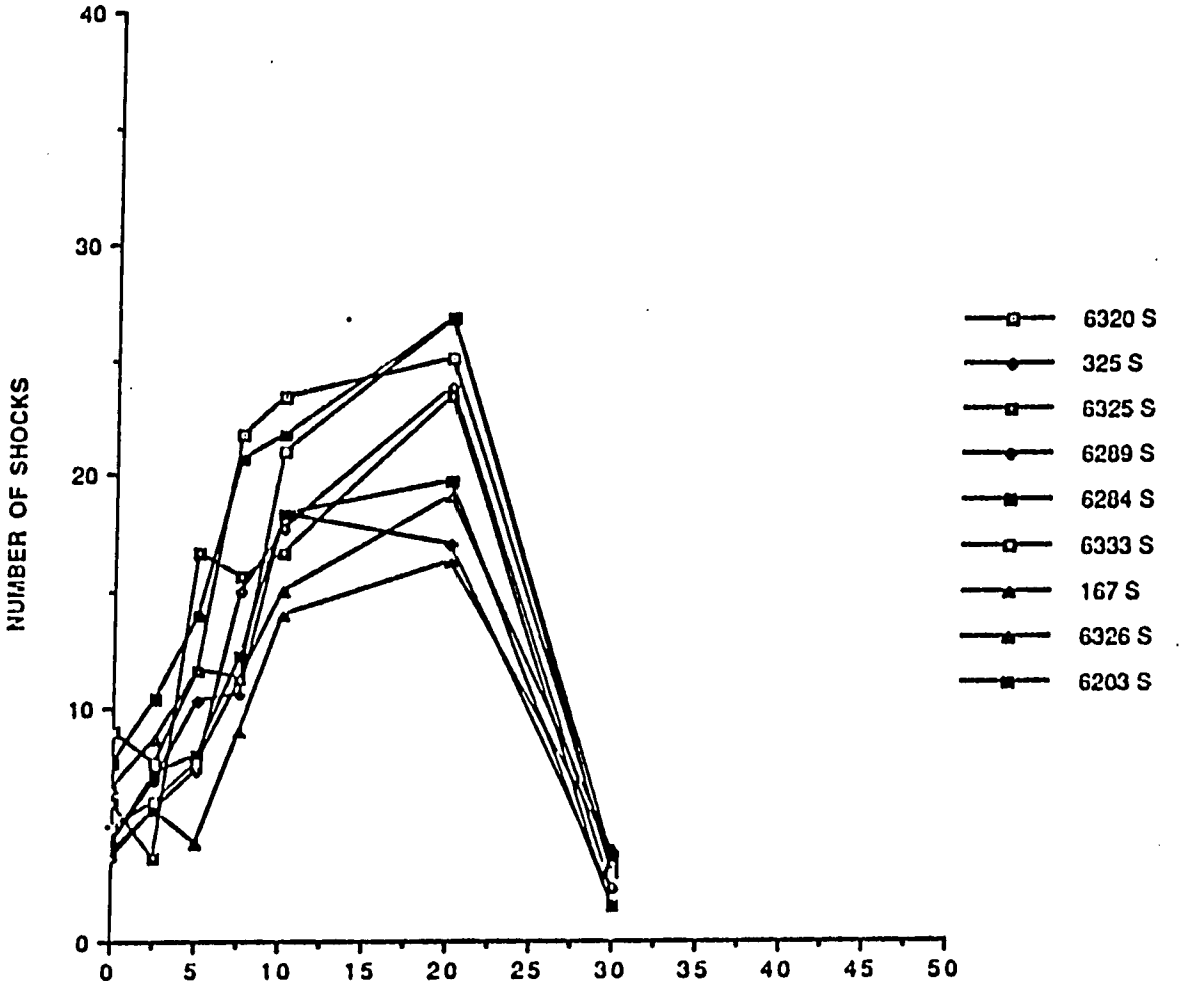


Figure 3

Mean Dose Response Profiles
Control, Drug Resistant and Drug Sensitive mice

Graphic representation of the group mean dose response curves. The curves indicate a shift to the right for drug resistant mice as compared to drug sensitive and control animals.

Figure 3

MEAN SHOCKS RECEIVED VS. DOSE

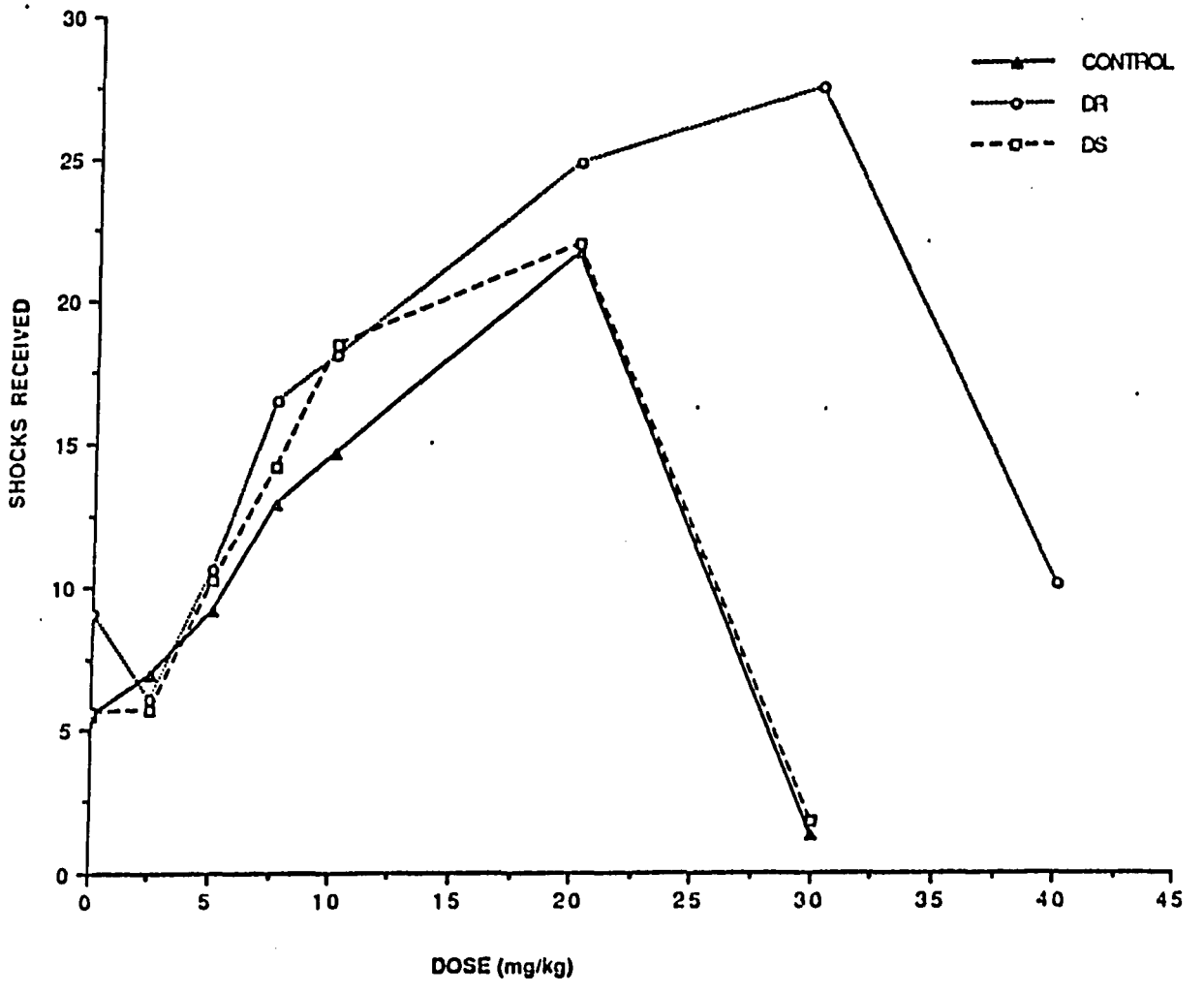


Figure 4

Percent of Baseline Responding VS Dose
Control, Drug Resistant and Drug Sensitive Mice

The complete dose response profile was divided into the anxiolytic and sedative phase. The graph illustrates the semi-linear dose functions generated by graphing the dose response data as percent of baseline responding. This mathematical translation compensates for differences which may exist in baseline responding.

Figure 4

PERCENT OF BASELINE RESPONDING VS DOSE

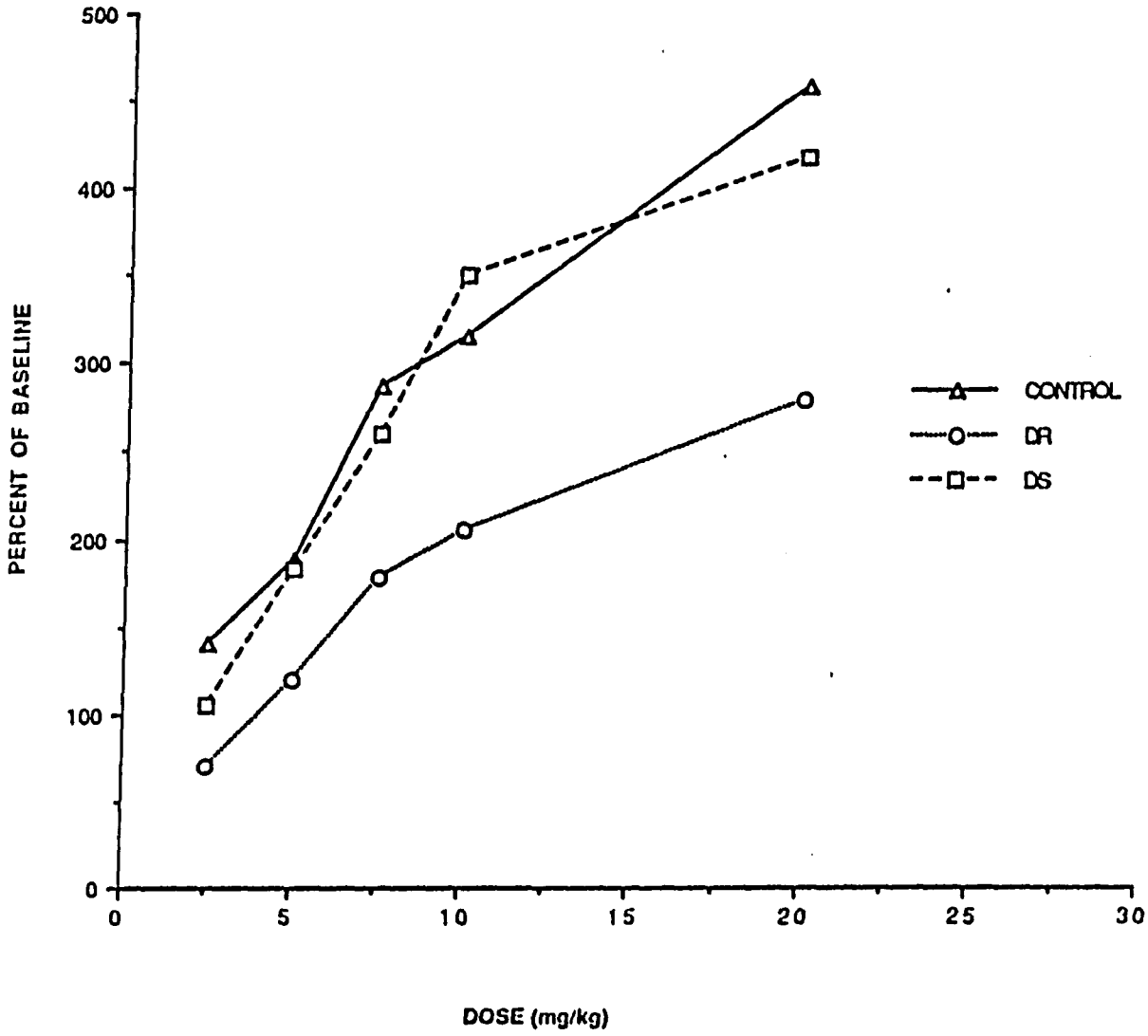


Figure 5

Log Percent of Baseline Responding VS Dose
Control, Drug Resistant and Drug Sensitive Mice

Log values were taken of the percent of baseline data, across all three strains, in order to further linearize the dose response functions generated in Figure 4.

Figure 5

LOG (PERCENT OF BASELINE) VS. DOSE

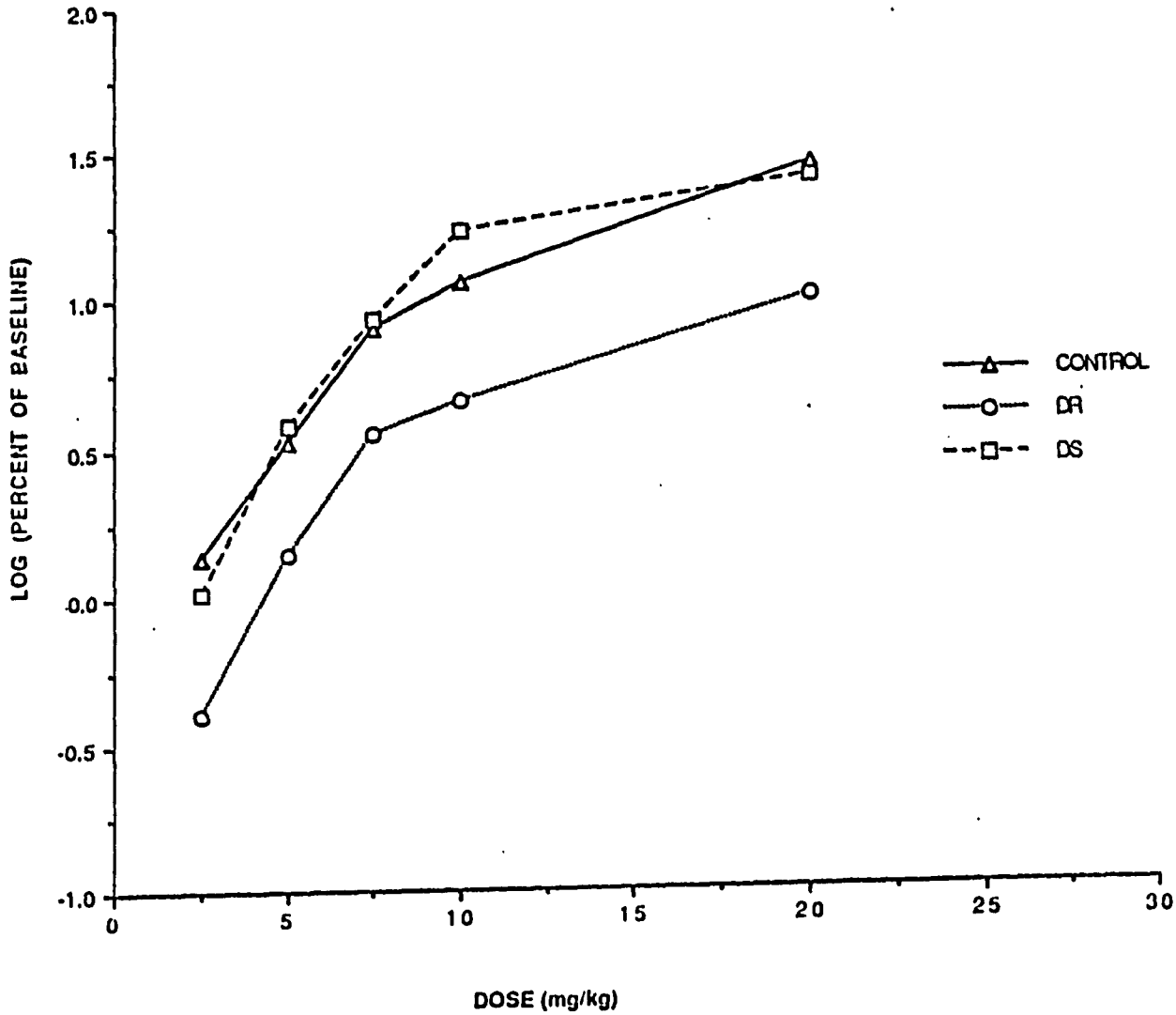


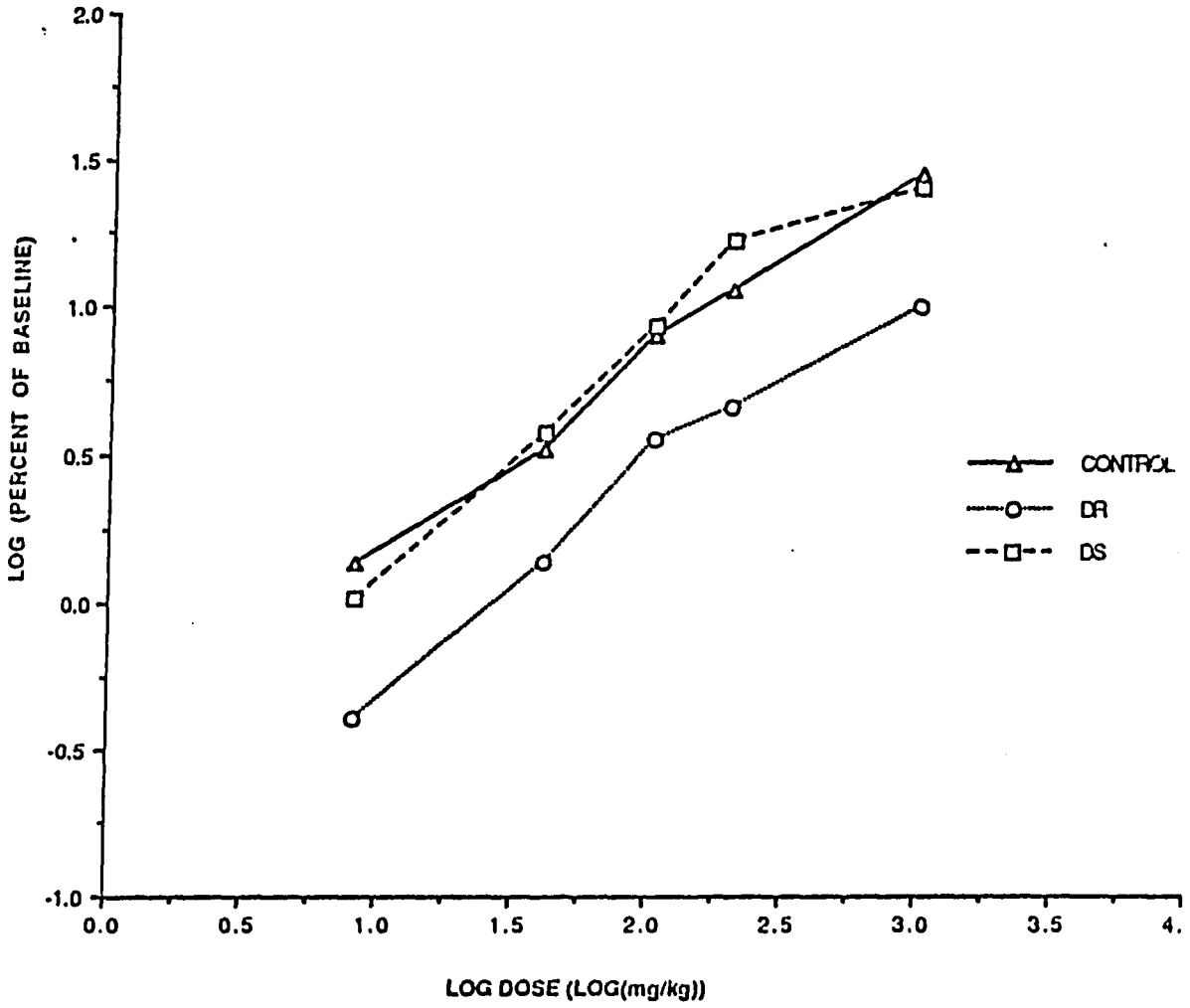
Figure 6

Log Percent of Baseline Responding VS Log Dose
Control, Drug Resistant and Drug Sensitive Mice

The log values for percent baseline responding were graphed against the log of the corresponding doses of chlordiazepoxide. The resultant functions are highly linear and allowed for the calculation of linear approximations to these mean percent of baseline dose functions.

Figure 6

LOG (PERCENT OF BASELINE) VS. LOG DOSE



in order to effect the same level of response as in the drug sensitive and control strains.

Approximate doses in the 2.5 mg/kg to 20 mg/kg range are based on a linear fit to the profiles shown in figure 5. For all three groups, a linear fit provided a good description of the observed dose-response profile over this range (response and dose both being expressed on the log scale). In fact, for the resistant group, linearity of the observed log dose - log response profile extended up to and including 30 mg/kg of diazepam. For consistency, calculations summarized in Tables 2a and 2b are based only on the 2.5 to 20 mg/kg range. Inclusion of the 30 mg/kg dose in calculations for the drug resistant group does not change the net results.

To quantify this apparent difference more precisely, a straight line was fit, by the method of least squares, to the response profile for each animal. The resulting lines were used to estimate, for each subject, the amount of drug required to achieve a specific percent increase in response over baseline responding. This was calculated for response levels corresponding to a 50%, 100% and 200% increase over baseline. The estimated dose required to effect a specific level of response was compared among the three groups using a one-way analysis of variance (Snedecor and Cochran, 1980). Table 2a shows the estimated dose of chlordiazepoxide required to produce an increase in response over baseline corresponding to 50%, 100% and 200% for each strain. In the drug resistant group, however, nearly twice the amount of chlordiazepoxide is needed to effect the same percent increase over baseline. This ratio is reasonably consistent across levels of response, as would be expected from the nearly parallel profiles demonstrated in Figure 4. Table 2b provides a statistical comparison, based on an ANOVA, of the doses required to effect a specific percent increase in response, comparing dose across strain. No significant differences were found between the control and drug sensitive groups ($p \leq .49$). For the resistant group,

Table 2a

Behavioral Effects of Librium
Estimated Dose Required to effect a Specific
Increase Over Baseline

	50% Increase	100% Increase	200% Increase
Control	3.17	5.57	12.35
Sensitive	3.80	5.87	10.81
Resistant	7.21	11.41	21.83

Estimated doses are given in mg/kg.

Table 2b

Behavioral Effects of Librium
Comparison of Dose Required to Effect a Specific Response
P-Values for Comparing Doses Across Groups

			50% Increase Over Baseline	100%Increase Over Baseline	200%Increase Over Baseline
Control	**vs.**	**Sensitive**	0.49	0.84	0.64
Control	**vs.**	**Resistant**	0.005	0.01	0.05
Sensitive	**vs.**	**Resistant**	0.03	0.02	0.02

Figures in the body of this table are p-values for testing whether the required dose is equal in both groups.

however, the amount of chlordiazepoxide required to achieve a specified increase in response was significantly higher ($p \leq .03$) than in either the DS or control strains.

Effects at the 30 mg/kg dose:

Subjects in the control and drug sensitive groups showed a very large decrease in response at the 30 mg/kg dose. most subjects in the resistant group, however, maintained an increased response at the 30 mg/kg dose (Figure 2), with a decrease in response manifested only at a 40 mg/kg dose level. It is evident from Figure 5 that the average response at 30 mg/kg was virtually identical in the control and drug sensitive groups, while that in the resistant group was significantly higher ($p \leq .001$). For completeness, however, a one-way analysis of variance of the log-transformed percent of baseline and the appropriate paired t-test were performed (Snedecor and Cochran, 1980).

Table 3 provides the statistical comparison of significant differences at 30 mg/kg across the strains. No statistically significant difference in mean response was identified between the control and drug sensitive groups. The resistant group, however, exhibited a significantly ($p \leq .0001$) higher mean response than either the sensitive or control groups. For that group, the response at 40 mg/kg was considerably lower, indicating that the change from anxiolytic to sedative effects occurred somewhere between 30 and 40 mg/kg for the drug resistant animals.

Table 3

Behavioral Effects of Librium
Results Observed at 30 mg/kg

3a) Means by Strain (Response = % of Baseline)

Strain	Average Response Log Units	Backtransformed Average
Control	-1.372	25% of baseline
Resistant	1.147	315% of baseline
Sensitive	-1.275	28% of baseline

3b) P-values for Testing Selected Comparisons

Comparison			P-value
Control	vs.	**Sensitive**	P = 0.73
Control	vs.	**Resistant**	P ≤.0001
Resistant	vs.	**Sensitive**	P ≤. 0001

P-values are based on two tailed t-tests, following a one-way analysis of variance of the log-transformed percent of baseline.

Potentiation of Ethanol by Chlordiazepoxide:

A dose of 3 g/kg ethanol induced narcosis in the DS and DR mice (Tables 4a and 4b) and the average sleep time was 45.4 and 45.1 minutes respectively. This difference in duration of narcosis between the two strains was not significant. Since benzodiazepines significantly potentiate the effects of ethanol, a dose response profile was run in order to profile differential strain effects.

For these data a simple descriptive straight line fit was performed by the method of least squares. While a plot of the response (sleep time) against dose approximated a linear function (Figure 7A), applying a log transformation to the dose, improved the degree of linearity (Figure 8). Figure 7B presents the graphic represention of data in Figure 7A in bargraph format in order to clearly illustrate the divergence in strain response to ethanol. Accordingly, the observed response data were analyzed by means of a straight line, fit to the sleep time verses log dose data. A separate line was fit, by the method of least squares for each strain. In these calculations the zero dose was adjusted when taking logs in order to render equal spacing of the control and two lowest doses on the log scale (Tukey, et al., 1985).

Table 5 summarizes the dose-response calculations for these data. The slope of the fitted log dose-response line was steeper for the sensitive than for the resistant strain (37.1 as opposed to 25.1). A test for nonparallelism shows that the slopes differed significantly at the 95% level. For the sensitive mice, a doubling in the dose resulted in an estimated increase in mean sleep time of 25.7 minutes. The corresponding estimated increase for the resistant group was only 17.4 minutes.

Librium-Ethanol Interaction in Trained Mice

As shown in Table 5, the mouse farthest removed from the average in the resistant group conflict dose-response function has a sleep time approximately 1.9 standard deviations away from the

Table 4a

Librium Potentiation of Ethanol - Raw Data
Dose-Response Data (Response = Duration of Sleep in Minutes)

Dose of Librium:	0 mg/kg	2.5 mg/kg	5.0 mg/kg	10.0 mg/kg	20 mg/kg
Resistant	46	27	62	55	150
	43	70	71	54	130
	66	25	70	85	80
	27	55	56	80	110
	60	65	50	107	130
	45	32	70	115	120
	43	45	74	71	96
	45	38	68	90	
	31	65	70	72	
Sensitive	42	46	100	95	105
	66	35	42	122	200
	28	85	42	63	190
	41	76	115	92	141
	62	40	107	95	172
	32	49	46	122	201
	27	61	77	95	141
	52	34	95	75	109
	50	68	105	90	
	54	55	80	114	

Table 4b

Librium Potentiation of Ethanol in Behaviorally Trained Mice. Dose-Response Data (Response = Duration of Sleep in Minutes) 10 mg/kg Librium + 3 g/kg ethanol

Resistant	Sensitive	Control
63	150	153
100	147	120
70	100	132
120	130	116
64	145	106
80	116	138
71	115	
	65	

Table 5

Librium Potentiation of Ethanol
Dose - Response Calculations

Resistant Mice:

Estimated Dose Response: Sleep Time (minutes) = 29.6 + 25.1 Log_e (Dose)

RMSE (Root Mean Square Error) from Regression = 18.3
Standard Error of Slope Estimate = 2.9

Predicted sleep time at 10 mg/kg Librium = 87.5 minutes
95% confidence limits on the predicted sleep time
for a single animal receiving 10 mg/kg = (49.9, 125.1)

Sensitive Mice:

Estimated Dose Response: Sleep Time (minutes) = 26.7 + 37.1 Log_e(Dose)

RMSE (Root Mean Square Error) from Regression = 27.0
Standard Error of Slope Estimate = 4.1

Predicted sleep time at 10 mg/kg Librium = 112 minutes
95% confidence limits on the predicted sleep time
for a single animal receiving 10 mg/kg = (56.7, 167.3)

Figure 7A

Chlordiazepoxide Potentiation of Ethanol
Drug Resistant and Drug Sensitive Mice

Loss of righting is measured in minutes by the duration of sleep induced at multiple doses of chlordiazepoxide, administered in the presence of 3 g/kg absolute ethanol. The graph indicates significant potentiation in sleep time for the drug sensitive mice at 20 mg/kg chlordiazepoxide.

FIGURE 7A
CHLORDIAZEPOXIDE POTENTIATION OF ETHANOL
INDUCED LOSS OF RIGHTING REFLEX

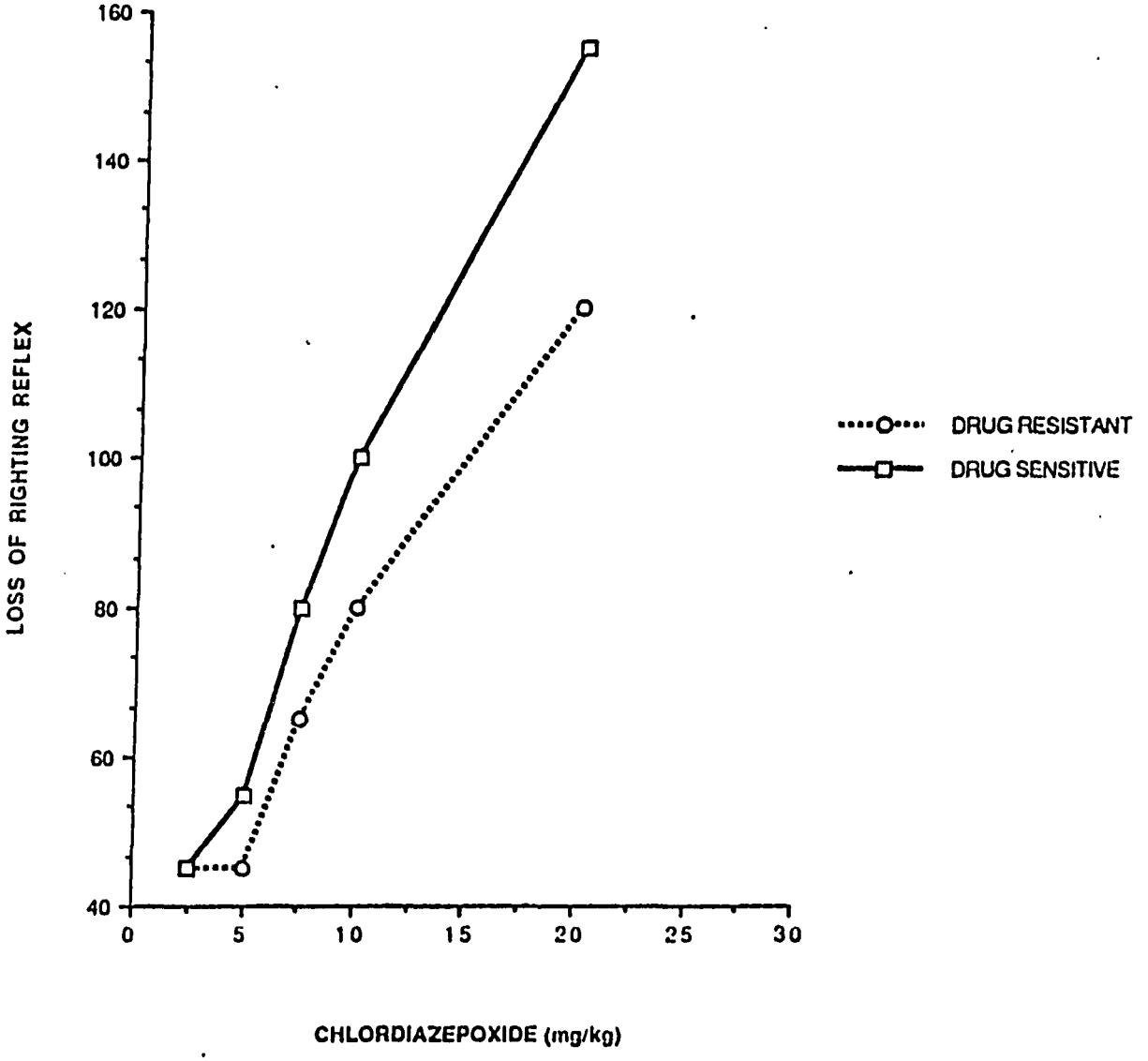


Figure 7B

Chlordiazepoxide Potentiation of Ethanol
Drug Resistant and Drug Sensitive Mice

Loss of righting reflex is measured as total sleep time (min.) The bar graph characterizes significantly greater sleep times for the drug sensitive mice. This trend is generalized across all doses of chlordiazepoxide tested, despite insignificant differences in baseline (saline) sleep times.

FIGURE 7B
CHLORDIAZEPOXIDE
POTENTIATION OF ETHANOL

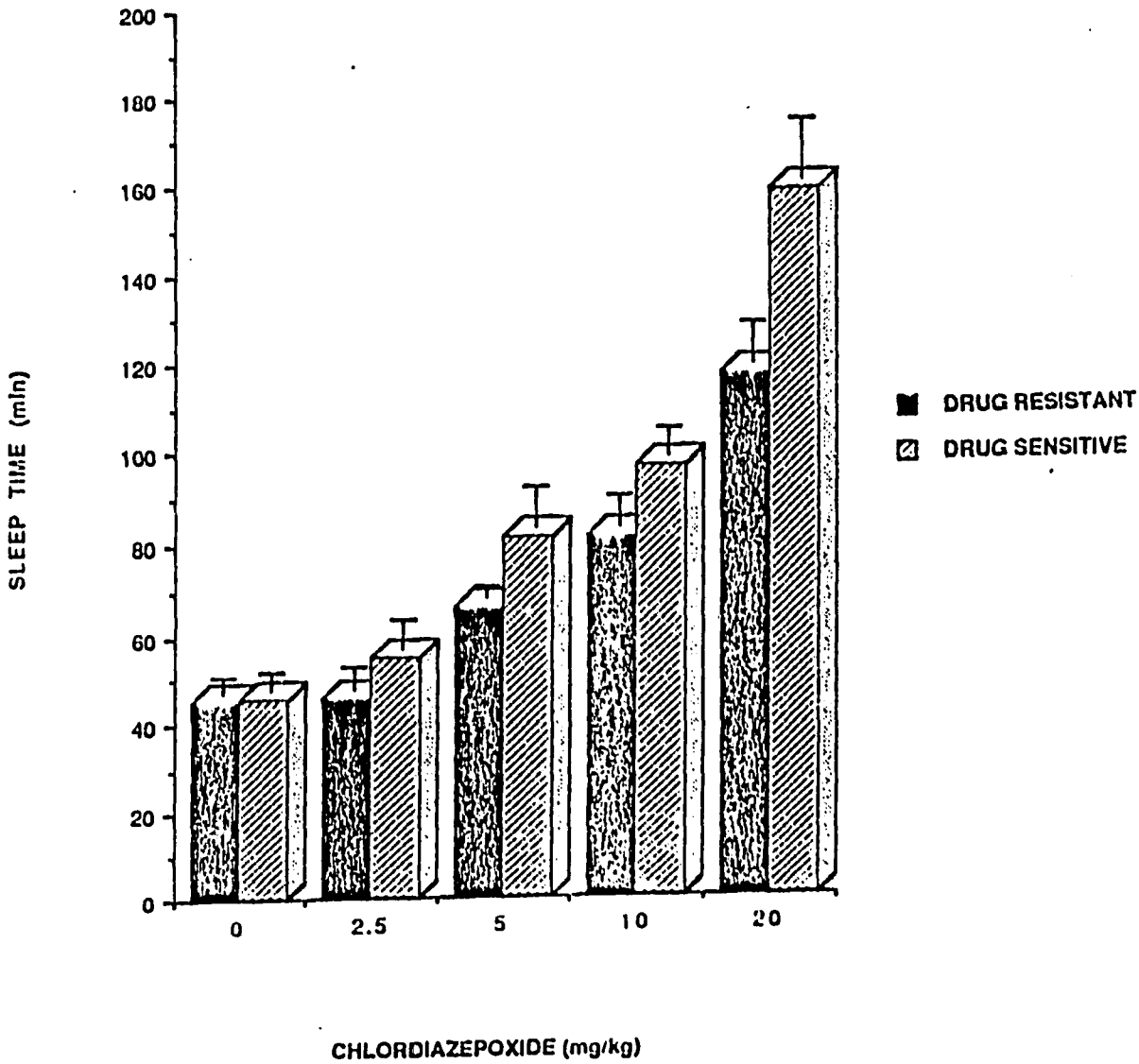
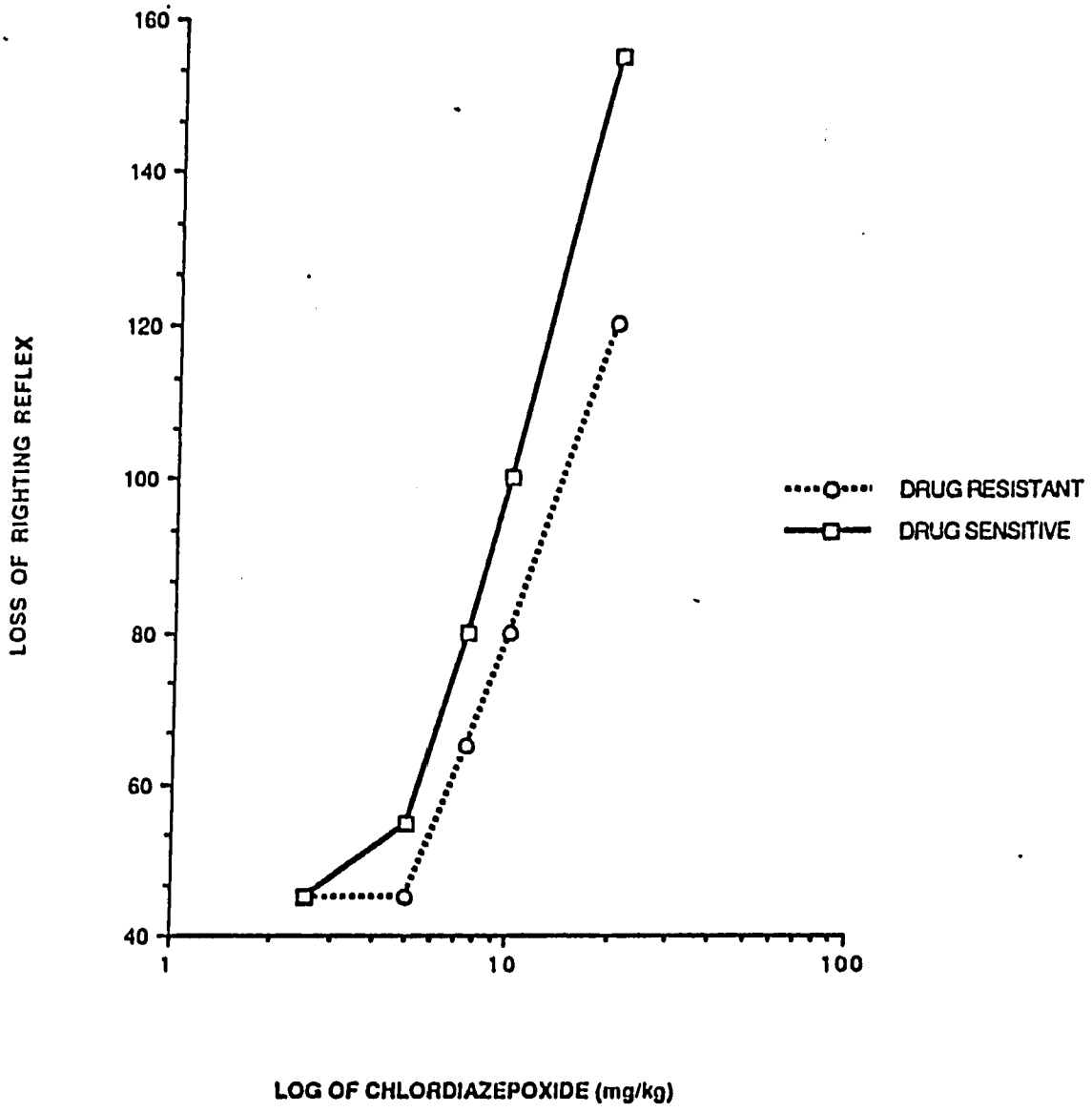


Figure 8

Chlordiazepoxide Potentiation of Ethanol (Log plot)
Drug Resistant and Drug Sensitive Mice

Log transformations of the dose profile were performed in order to further linearize the potentiation functions for each strain (DR, DS). The drug sensitive profile is steeper and shifted to the left suggesting that lower doses of chlordiazepoxide are necesary to potentiate longer sleep times. The drug resistant function demostrates resistancy toward the sedative cumulative effects of ethanol and chlordiazepoxide as depicted by shorter sleep time.

FIGURE 8
CHLORDIAZEPOXIDE POTENTIATION OF ETHANOL INDUCED LOSS OF RIGHTING REFLEX



mean. The same is true for the sensitive mice. When the 95% confidence limits are considered none of the animals fall outside this range.(Table 5)

As shown in Table 7, the control mice given 10 mg/kg chlordiazepoxide plus 3 g/kg ethanol, slept for 127.5 min. The sensitive and resistant mice slept 121.6 and 81.4 min. respectively. No statistically significant differences in average sleep time were identified between the control and sensitive groups ($p \leq .661$). The resistant group however exhibited a significantly shorter average sleep time than the control mice ($p \leq .001$) or the sensitive mice ($p \leq .001$).

Table 6

Librium Potentiation of Ethanol
Summary Statistics for Mice Previously
Tested in Behavioral Protocol

RESISTANT MICE

Average Sleep Time	=	81.4 minutes
Standard Deviation	=	20.4 minutes

Most extreme sleep time = 120 minutes (1.89 standard deviations above the mean)

SENSITIVE MICE

Average Sleep Time	=	121.6 minutes
Standard Deviation	=	27.4 minutes

Most extreme sleep time = 70 minutes (1.88 standard deviations above the mean)

Table 7

Librium Potentiation of Ethanol
Observed at 10 mg/kg

Means by Strain:

Strain	Average Sleep Time (min)
Resistant	81.4
Sensitive	121.6
Control	127.5

P-Values For Tested Selected Comparisons:

Comparison			P-Value
Control	**vs.**	**Sensitive**	P ≤.661
Control	**vs.**	**Resistant**	P ≤.001
Resistant	**vs.**	**Sensitive**	p ≤.001

P-Values are based on two tailed t-test.

BIOCHEMICAL RESULTS:

[^{3}H]Flunitrazepam Binding in the Presence and Absence of GABA:

BDZ receptor activity was assayed for tissues from three brain regions (cortex, cerebellum and hippocampus) across the DS, DR and control mice. Individual B_{max} and K_d values for the various brain regions across strain are presented in Table 8a. Data from the second assay, investigating the stimulatory effect of GABA on BDZ binding, are provided in Table 8b. For each of the four parameters of interest (B_{max}/K_d in the presence and absence of GABA), and for each brain region, a one-way analysis of variance (ANOVA) was performed with strain as a factor. The ANOVA allowed the attainment of a pooled estimate of the random variation in the data, combining information from all three strains. Specific pairwise comparisons of interest among strains were made using the paired t-test, with the mean square error from the ANOVA in the error term.

Tables 9 and 10 summarize results for K_d and B_{max} determinations respectively. Table 9 reveals that receptor affinity (K_d) in the cortex does not differ significantly between the resistant and sensitive mice, both in the presence ($K_d \approx 1$ nM) and absence ($K_d \approx 0.4$ nM) of GABA. The K_d values for the drug sensitive and resistant strains were also similar in the hippocampus ($K_d \approx 2$ nM). Significant strain divergence did exist for the K_d values generated for the cerebellar tissue ($p \leq .044$) in the absence of GABA for DS and DR mice ($K_d = 2.06$ and 1.54 nM, respectively). K_d values for the control strain were significantly ($p \leq .001$) higher than the resistant or sensitive strains in both the cortex and cerebellum. In the presence of GABA, K_d decreased in all regions across the three strains tested (DR, DS and albino). However, there was little difference in the affinity constant (K_d) across the DS and DR mice, in the presence of GABA. The control albino cortical samples were still significantly higher ($p \leq .001$) than either the resistant or sensitive strains. The statistically significant

Table 8a

Flunitrazepam Binding Assay
Raw Data (minus GABA)

Strain	K_d (nM)			B_{max} (fmoles/mg protein)		
	Cortex	Cerebellum	Hippocampus	Cortex	Cerebellum	Hippocampus
Control	3.075	2.193	-	2975.8	1255.8	-
Control	2.712	2.986	-	3918.1	1160.1	-
Control	2.151	3.237	-	3729.6	2118.8	-
DR	0.878	2.028	1.752	1814.7	1466.8	2317.4
DR	0.986	1.951	2.156	1816.3	1215.3	1968.2
DR	1.038	2.193	1.983	1603.9	2185.6	2051.9
DR	0.948	-	1.835	1422.8	-	1514.7
DR	0.866	-	1.623	1387.8	-	2008.4
DR	1.047	-	1.893	1432.0	-	2355.0
DS	1.119	1.793	1.846	1628.2	1726.4	1490.9
DS	0.939	1.457	2.063	1887.6	1762.5	1482.2
DS	0.890	1.536	1.972	1336.2	2024.0	1519.2
DS	0.844	1.400	1.863	1587.4	1822.2	1996.0
DS	0.931	1.492	2.327	1588.0	2416.8	2621.8
DS	0.951	1.472	1.671	1499.5	1952.6	1798.1

DR = Drug Resistant
DS = Drug Sensitive

Table 8b

Flunitrazepam Binding Assay
Raw Data (Plus GABA)

Strain	K_d (nM)			B_{max} (fmoles/mg protein)		
	Cortex	Cerebellum	Hippocampus	Cortex	Cerebellum	Hippocampus
Control	0.589	1.291	-	3118.8	984.5	-
Control	1.197	1.413	-	4687.0	1169.8	-
Control	0.897	0.878	-	3690.2	1398.7	-
DR	0.456	1.269	0.997	2219.8	1820.2	2442.2
DR	0.391	0.957	1.278	2089.7	1534.1	1894.4
DR	0.350	1.135	0.855	1632.6	2479.1	2204.4
DR	0.394	-	1.122	1686.0	-	1773.5
DR	0.439	-	1.057	1588.4	-	2052.8
DR	0.503	-	0.974	1617.25	-	2530.2
DS	0.328	1.088	1.525	1812.4	1978.2	1974.7
DS	0.389	0.760	1.389	2120.1	1802.6	1568.9
DS	0.360	1.380	1.220	1787.9	2571.7	1823.0
DS	0.369	0.779	1.067	1681.4	2514.9	2040.2
DS	0.419	0.877	0.812	1835.2	1998.4	1938.8
DS	0.474	0.926	1.006	1172.5	2268.7	1923.5

DR = Drug Resistant
DS = Drug Sensitive

Table 9

Flunitrazepam Binding Assay
Mean K_d (nM) Values by Strain and Tissue

1a) No GABA Present in the Assay:

	Cortex	Cerebellum	Hippocampus
Control	2.65	2.81	-
Resistant	0.96	2.06	1.87
Sensitive	0.95	1.54	1.96
(Root Mean Square Error)	(0.21)	(0.30)	(0.21)

P-Values for selected comparisons (2-sided t-test, unadjusted for multiple Comparisons)

Control	vs.	**Resistant**	≤.0001	.015	-
Control	vs.	**Sensitive**	≤.0001	.0004	-
Sensitive	vs.	**Resistant**	≤.90	.044	.50

1b) GABA Present in the Assay:

	Cortex	Cerebellum	Hippocampus
Control	0.89	1.19	-
Resistant	0.39	0.98	1.05
Sensitive	0.39	0.98	1.17
(Root Mean Square Error)	(0.13)	(0.24)	(0.21)

P-Values for selected comparisons (2-sided t-test, unadjusted for multiple comparisons)

Control	vs.	**Resistant**	≤.0002	.72	-
Control	vs.	**Sensitive**	≤.0003	.26	-
Sensitive	vs.	**Resistant**	≤.68	.44	.34

Table 10

Flunitrazepam Binding Assay
Mean B_{max} (Fmoles/mg protein) Values by Strain and Tissue

1a) No GABA Present in the Assay:

	Cortex	Cerebellum	Hippocampus
Control	3541.2	1511.5	-
Resistant	1579.6	1622.6	2035.9
Sensitive	1587.8	1950.4	1818.1
(Root Mean Square Error)	(266.9)	(416.8)	(380.2)

P-Values for selected comparisons (2-sided t-test, unadjusted for multiple comparisons)

Control	**vs. Resistant**	≤.0001	.75	-
Control	**vs. Sensitive**	≤.0001	.19	-
Resistant	**vs. Sensitive**	.96	.31	.34

1b) GABA Present in the Assay:

	Cortex	Cerebellum	Hippocampus
Control	3832.0	1184.3	-
Resistant	1805.6	1944.5	2149.6
Sensitive	1734.9	2173.1	1878.2
(Root Mean Square Error)	(420.9)	(360.1)	(242.8)

P-Values for selected comparisons (2-sided t-test, unadjusted for multiple comparisons)

Control	**vs. Resistant**	≤.0001	.03	-
Control	**vs. Sensitive**	≤.0001	.006	-
Resistant	**vs. Sensitive**	.78	.41	.08

($p \leq .05$) strain differences observed in the cerebellum, in the absence of GABA, were no longer evidenced in the presence of GABA ($K_d \approx 1$ nM.). While the receptor affinity values did indicate significant difference between the resistant and sensitive lines, a significant strain difference was apparent between the HS/IBg bred DS and DR mice and the albino controls.

The trends in the B_{max} values were quite similar to the K_d values in the cortex and hippocampus (Table 10). The B_{max} values in the cortex for the control strain were greater than B_{max} values for the resistant and sensitive mice (3.5 pmole/mg protein vs 1.5 pmole/mg protein). B_{max} values for the sensitive and resistant strains were comparable in the cortex, cerebellum and hippocampus. Statistical significance was approached ($p \leq .08$) for the B_{max} values computed in the hippocampus for DS verses DR mice. Since GABA effects only the affinity of the BDZ receptor for agonists, no changes in B_{max} were observed in the presence of GABA (Table 10)

[^{35}S]TBPS Binding in DS and DR Cortex

Five cortical tissue samples from resistant mice and four from drug sensitive animals were assayed. Specific binding of the radiolabeled ligand [^{35}S]TBPS was obtained for each sample in the absence of diazepam, and in the presence of ten concentrations of diazepam, ranging from .00316 to 100 μM. Specific binding at the different diazepam concentrations was expressed as a percentage of specific binding in the absence of diazepam. The transformed data are shown in Table 11.

A repeated measures analysis (Morrison, 1976) was used to compare dose-response profiles across the two behavioral strains. The analysis examined whether the average profiles in both groups were similar in shape and level. The test for strain-by-dose interaction in the analysis addressed the issue of possible shape differences in the response profiles. The absence of such an interaction corresponded to parallel profiles for both strains,

Table 11

Diazepam Potentiation of TBPS Binding in Cortical Tissue
Raw Data for Tissue Samples from Resistant and Sensitive Mice

Drug Resistant Group: Specific Binding as Percent of Baseline

Concentration of Diazepam(nM)	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
0.00316	132.6	168.7	238.9	168.5	235.7
0.01	144.1	174.1	284.8	158.7	221.0
0.0316	172.2	179.6	310.0	177.9	229.6
0.10	181.9	194.7	315.2	204.9	260.6
0.316	166.4	198.5	282.7	189.9	246.6
1.00	188.5	220.6	304.3	222.2	261.0
3.16	192.9	184.7	256.8	193.2	224.7
10.00	198.9	206.4	315.2	193.1	226.3
31.60	205.6	206.5	263.8	190.0	229.4
100.0	195.1	172.7	225.5	160.5	205.3

Drug Sensitive Group: Specific Binding as Percent of Baseline

0.00316	129.8	125.7	156.8	322.5
0.01	155.1	129.1	156.9	332.1
0.0316	159.5	151.4	176.5	317.9
0.10	174.2	152.9	171.8	317.9
0.316	165.9	143.7	182.5	349.8
1.00	165.1	163.6	179.1	389.1
3.16	160.6	146.6	165.8	341.9
10.00	161.6	136.1	158.7	298.6
31.60	145.1	128.7	147.2	288.4
100.0	140.1	120.4	131.3	236.1

indicating a difference in response between strains (Figures 9, 10), consistent across doses.

In the absence of an underlying theoretical model to describe the dose response profiles shown in Figure 10, a characterization of these profiles may be carried out by means of a regression analysis of the repeated measures data (Yates, 1982). In the A-L-Q analysis, the average term represents a measure of the mean response level over all doses. The linear effect measures the overall trend for that dose. The trend can be operationally defined as the average rate of increase/decrease for responses at a given dose. A significant difference between strains in this component would indicate a difference in the rate of linear increase/decrease in response. The quadratic component in an A-L-Q analysis provides a measure of the curvature in the overall dose response. A positive value indicates concavity while a negative value identifies a convex function. A significant strain difference in the quadratic term would indicate a difference in the average degree of concavity/convexity for the response profiles.

Figure 9 shows the average dose-response profile for both strains. The overall repeated measures analysis showed no statistically significant strain by dose interaction ($p \leq .10$), indicating no severe nonparallelism of the response profiles. Analysis of the individual A-L-Q components is summarized in Tables 12-14. In each of these tables, the degree of freedom, corresponding to "strain," test for consistency of that component across strains. In Table 13 and 14, the degree of freedom for "mean" is associated with the test that the component is zero. Thus, p-values for testing mean linear and quadratic components ($p \leq .64$ and $p \leq .003$, respectively) indicate that the linear component of the dose-response profile is effectively zero in both groups, while a strong nonzero quadratic component exists. Those results are not surprising, given the parabolic shape of the observed dose-response profiles. P-values for testing consistency of the linear and quadratic components across both strains ($p \leq .15$ and $p \leq .58$, respectively) indicate no statistically significant strain differences. Table 12 indicates that the difference between

Figure 9

Diazepam Potentiation of TBPS Binding (strain averages)
Drug Resistant and Drug Sensitive mice

Specific bound counts are represented as percent of baseline. The strain averages are represented in two functions. The drug resistant profile is consistantly greater than the drug sensitive profile, suggesting some possible differential effects in chloride ion interaction within the supramolecular complex, across the DR and DS mice.

Figure 9

TBPS Binding Data: Average Response vs. Dose

Response = Specific Bound Count as % of Baseline

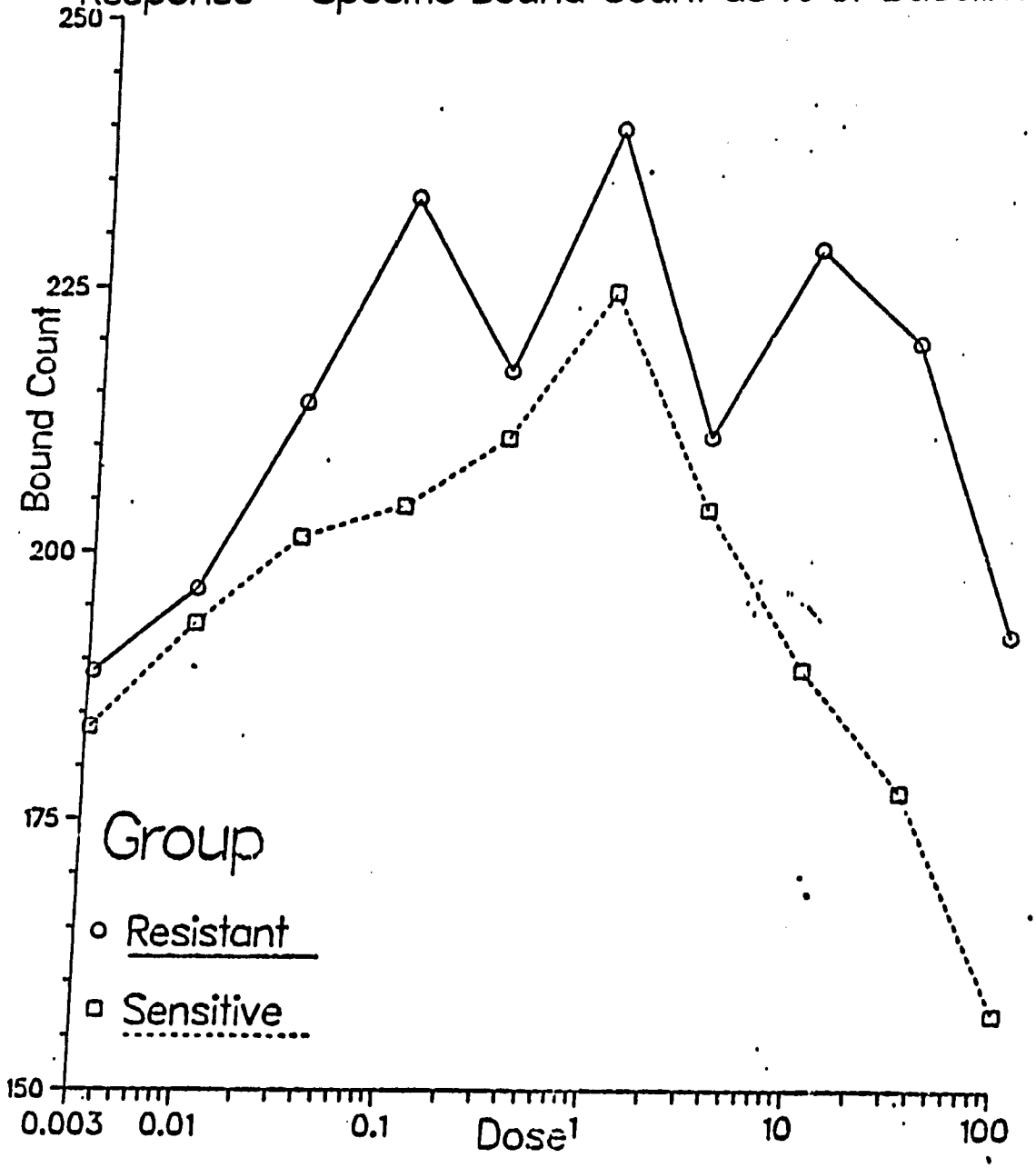


Figure 10

Diazepam Potentiation of TBPS Binding
Individual Dose-Response Profiles
Drug Resistant and Drug Sensitive mice

Specific bound counts are represented as percent of baseline. The bound counts for the drug resistant sample are significantly greater than all but one profile in the drug sensitive group. The validity of this one drug sensitive sample in accurately measuring diazepam potentiation of TBPS binding is therefore compromised. Elimination of this one measurement permits a clear and significant distinction to be made between the two strains.

Figure 10

TBPS Binding Data: Individual Dose–Response Profiles
(Response = % of Baseline Bound Count)

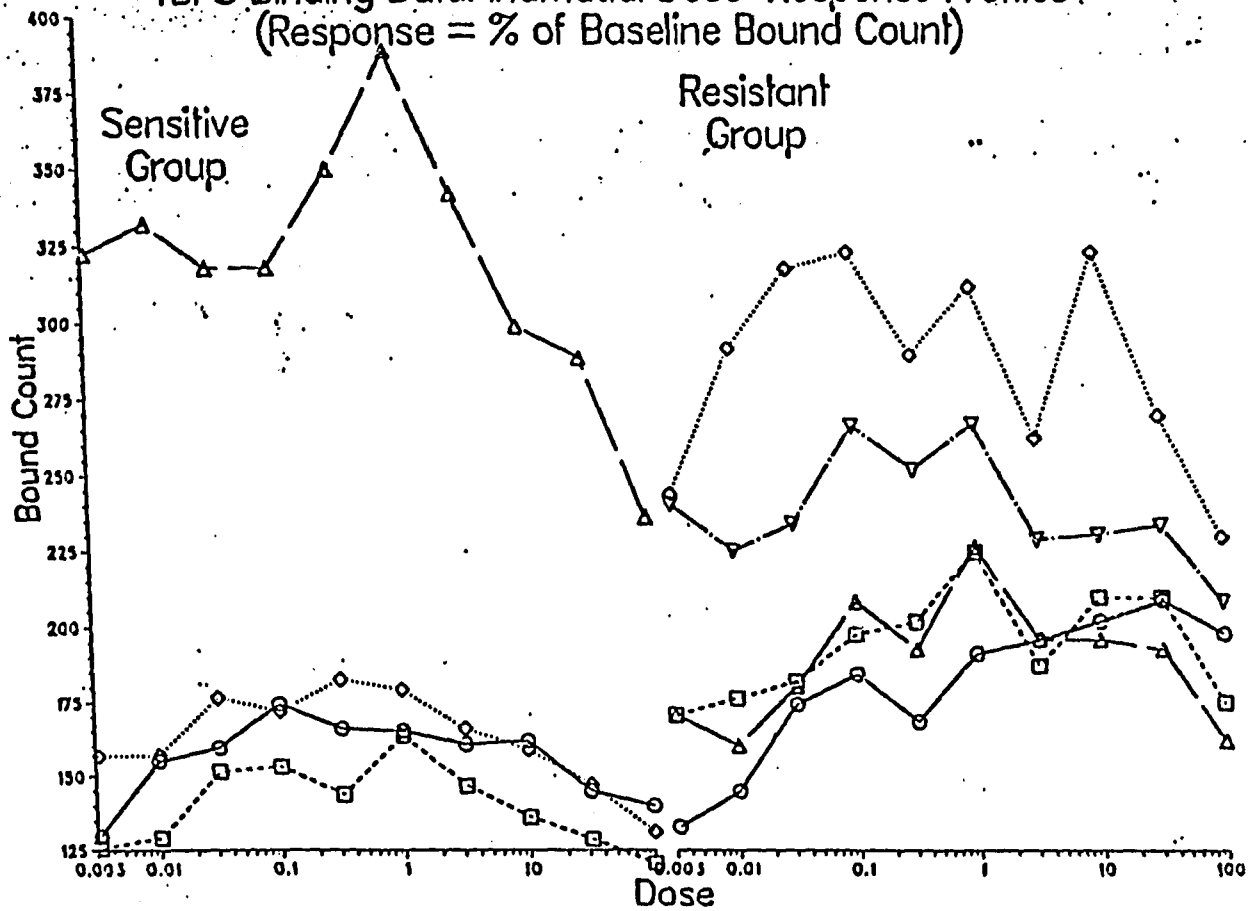


Table 12

Diazepam Potentiation of TBPS Binding in Cortical Tissue
A-L-Q Analysis: Results for 'Average' Component

1a) ANOVA Table:

Source of Variation	Degrees of Freedom	Mean Square	F-Value	P-Value
Strain	1	819.2	0.20	0.67
Error	7	4066.8		

1b) Mean of the 'Average' Components, by Strain

Strain	n	Mean
Resistant	5	213.6
Sensitive	4	194.4

(units are expressed as percent of baseline)

1c) Individual values of the 'Average' Component

Replicate	Resistant	Sensitive
1	177.8	155.7
2	190.7	139.8
3	279.7	162.7
4	185.9	319.4
5	234.0	

Table 13

Diazepam Potentiation of TBPS Binding in Cortical Tissue
A-L-Q Analysis: Results for the 'Linear' Component

ANOVA Table:

Source of Variation	Degrees of Freedom	Mean Square	F-Value	P-Value
Mean	1	.024	0.24	0.64
Strain	1	.257	2.58	0.15
Error	7	.100		

Table 14

Diazepam Potentiation of TBPS Binding in Cortical Tissue
A-L-Q Analysis: Results for the 'Quadratic' Component

ANOVA Table:

Source of Variation	Degrees of Freedom	Mean Square	F-Value	P-Value
Mean	1	1.84	44.2	0.0003
Strain	1	0.01	0.34	0.58
Error	7	0.04		

strains in the mean of this average level was not statistically significant ($p \leq .67$). Table 15 provides the respective concentrations of diazepam at which maximal stimulatory effects on [^{35}S]TBPS binding are realized for the DR and DS strains. The average concentration of diazepam at which maximal stimulation of [^{35}S]TBPS occurs in the DR and DS strains is 0.82 nM and 0.38 nM, respectively. These data when compared across strains are significantly different ($p \leq .06$).

Table 15

Diazepam Stimulation of TBPS Binding
Maximal Stimulatory Concentration of Diazepam
For Drug Sensitive and Drug Resistant Mice

Drug Resistant Concentration (nM)	**Drug Sensitive** Concentration (nM)
1.00	.10
1.00	.10
1.00	.32
1.00	1.00
.10	

DISCUSSION

BEHAVIORAL ANALYSIS:

A new repeat measures conflict procedure was developed in mice. By careful controlled manipulation of the positive and negative reinforcers it was possible, over several months, to design a valid, reliable and stable conflict procedure for mice. This procedure proved to generate accurate dose response functions and was found sensitive to benzodiazepine treatment. This conflict paradigm combined the stable baseline provided by Geller-like procedures with the simple acquisition of a licking response, utilized by Vogel et al. The advantages of such a baseline are its stability over time, low interanimal variability and it's ease of training. Long periods of positive reinforcement coupled with very clear presentation of shocked responses might account for the high stability in intersubject responding over time

Close examination of the dose response functions (Figure 3) reveals a separation between the drug resistant strain and the drug sensitive or control strains. The drug sensitive and control profiles are practically superimposable (Figure 2A and 2B) and would suggest little distinction in their anxiolytic response as mediated by the effects of chlordiazepoxide. Their dose response curves approximate standard dose related inverted U-shaped functions. The anxiolytic effect is potentiated, between 2.5 and 20 mg/kg of chlordiazepoxide, in proportion to the dose. The maximal effect is realized at 20 mg/kg for both sensitive and control strains. However, once the dose is increased past 20 mg/kg, a sharp decline in anxiolytic effect is evident. It is possible to conclude, based on the dose response profiles for the sensitive and control mice, that the anxiolytic component of the response exists between 2.5 and 20 mg/kg. Doses exceeding 20 mg/kg severely decrease shocked responding as well as free licking. The decrease in free licking rates serves as an accurate measure of sedation. It is not apparent, based on these data, that

the drug sensitive mice are more sensitive than controls to the anxiolytic effects of chlordiazepoxide. Direct comparison of the drug sensitive and control animals does not suggest any behavioral sensitivity, for the DS mice, toward the sedative effects of chlordiazepoxide. This conclusion is based on the 30 mg/kg dose which maximally decreases responses to 25% and 28% of baseline (Table 3), respectively, in both the control and drug sensitive strains.

Gallaher et al. (1987) reported significant differences across the DR, DS and control mice in their sensitivity to the sedative effects of diazepam. The data presented in these studies does not corroborate Gallahers' finding. However, Gallaher looked at the duration of the sedative effect. The conflict procedure developed and implemented in these studies measured the magnitude of the anxiolytic and sedative effects, at one single interval, between 30 and 45 min after administration. While Gallaher supported his findings with a direct measure of BDZ levels in DS and DR brain tissue, 30 min. after administration, the largest separation in sedative effect, in the two strains, occured approximately 300 min after administration. Therefore, the lack of significant sensitivity on the part of the DS mice, as measured by this novel conflict procedure, may reflect the magnitude of effect rather than duration. Future, investigation might include profiling brain BDZ levels, in the DS and DR strains, 300 min after administration of a known BDZ compound.

The dose response profile for the DR animals is similarly an inverted U-shaped function. This dose response profile, though, is significantly shifted to the right (Figure 3). The dose response functions are quite similar for the sensitive and control strains between 2.5 and 20 mg/kg chlordiazepoxide. Aside from the magnitude of increase in shocked responses, there is a real and substantiated dose related shift. The shift describes a significant increase in the dose of chlordiazepoxide at which optimal anxiolytic effects are realized. For the drug resistant group this dose is identified as 30 mg/kg compared with 20 mg/kg for both the sensitive and control groups. In addition, while 30 mg/kg is

the effective sedative dose for the control and drug sensitive mice, a dose greater than 40 mg/kg is necessary to produce maximal sedation. As Margules and Stein (1968) have shown, the sedative effects of BDZ's can suppress conflict responding and a resistance to the sedative effects of BDZ's in DR mice could account for the shift to the right in the dose response curve for DR mice. These studies present conclusive evidence to support the divergence in anxiolytic response across the drug resistant and drug sensitive strains. These data also substantiate the differential sensitivity, across strain, toward the sedative effects of benzodiazepines as reported by Gallaher et al. (1987).

Baseline levels of conflict responding in DR mice were somewhat higher than that observed for DS and control mice. When differences were studied, based on percent of baseline response, there was an apparent decrease in anxiolytic response to chlordiazepoxide by the DR mice (Table 2a). These differences, although significant, can be accounted for by differences in baseline response rates. The most parsimonious explanation for all of these data is that the DR mice are less sensitive to the sedative effects of BDZ's than control or DS mice. The resistant mice also showed greater variability in their conflict dose response curves than control or sensitive mice. This suggests that the inbreeding selection process favored this strain over control or sensitive mice.

A protocol was designed to test the strains response to the potentiation of ethanol by librium, in order to confirm the differential anxiolytic response across these divergent strains. These results were highly correlated with the behavioral data previously discussed. Independent verification in the classification of the drug resistant and drug sensitive mice was also rendered. Liljequist and Engel (1984) profiled the interactive anxiolytic effects of ethanol and diazepam. Their results highlighted the cumulative anxiolytic effect of these two compounds. In addition, their data points to a narrow anxiolytic dose range associated with ethanol, prior to sustaining secondary sedative effects.

The results confirmed the divergence in strains on yet another behavioral measure. The mean sleep time induced by 3 g/kg ethanol, did not differ across the drug resistant or drug sensitive mice. While one might expect to report a significant potentiation in sleep time among the drug sensitive strain, the data from the behavioral profile of diazepam would suggest no significant sensitivity toward the effects of diazepam. However, since the previous behavioral data did support a robust strain difference in response to diazepam, a comparison of the effects of benzodiazepines on the sedative properties of ethanol seemed most appropriate.

According to the data presented by Liljequist and Engel (1984), benzodiazepines enhance the sedative effects of ethanol. In line with that notion, the next set of studies profiled the differential effects of several doses of chlordiazepoxide on the sedative effects of ethanol. The data was in direct concurrence with the behavioral profiles. The drug resistant mice slept for a shorter duration than either the drug sensitive or control mice at each dose of chlordiazepoxide tested (Figures 7B). Because no significant difference in sleep time was shown between the respective strains, based on the effects at 3 g/kg ethanol alone, these data provide confirmation of the differential effects of chlordiazepoxide on the conflict behavior of the differential strains.

The data on the potentiating effects of chlordiazepoxide on ethanol were also implemented as an independent measure of the classification of the resistant and sensitive mice. The standard deviations of the mean were used to measure each animal's response compared to others in it's group. The issue arises, whether or not a particular mouse should be included in calculating the mean and the standard deviation for it's group. To circumvent this issue, one can utilize the independent dose-response data. The mice in question received 10 mg/kg chlordiazepoxide in the ethanol potentiation test. From the linear fit to the observed dose-response profile, a 95% confidence limit can be placed on the predicted value of a single observation at 10

mg/kg for each individual strain. If any mice from the behavioral testing paradigm has a sleep time falling outside these 95% limits, strong evidence for misclassification would exist. It is clear from an examination of Table 6 that the mouse farthest removed from the average, in the resistant group, has a sleep time approximately 1.9 standard deviations away from the mean. The same is true for the sensitive mice. These data, therefore, support the conclusion that all mice previously tested in the behavioral paradigm were indeed correctly classified as either resistant or sensitive. No differences were seen in chlordiazepoxide potentiation of ethanol for sensitive and control animals, which directly support the lack of difference in the anxiolytic responsiveness of these two strains in the conflict paradigm.

BIOCHEMICAL ANALYSIS:

Several biochemical measures were conducted on neural tissue extracted from ninth generation drug resistant and drug sensitive mice in addition to a control albino ICR strain. The intention of these measures was to investigate the possibility that biochemical differences exist, between the DR and DS mice, on a receptor level, and mediate the behavioral differentiation evident across these two divergent strains. K_d and B_{max} values were computed for separate regions, the cerebral cortex, hippocampus and cerebellum, across the strains. The K_d value represents a quantitative analysis of the affinity of the receptor for the tritiated ligand, which in this case was [^{3}H]flunitrazepam. The Bmax value describes the number of BDZ receptors present in the tissue assayed. Together, these two quantitative indices describe the state and density of the BDZ receptor population.

These data, however, did not generate significant differences across the drug resistant and drug sensitive samples. While the data suggested no difference in the BDZ receptor K_d and B_{max}, one might hypothesize that subtle differences exist in the dynamic interaction among the BDZ, GABA recognition sites and the chloride ion channel. The next logical progression was to profile the potentiating effects of GABA across these strains. Similarly, [^{35}S]TBPS binding (Skolnick et al., 1986) might highlight any alterations in chloride ion channel interactions within the supramolecular complex and was also warranted in providing a complete and thorough analysis of the mechanism of the supramolecular complex. Any evidence which suggested differential BDZ, GABA and chloride interactions across strain might then be functionally linked to the behavioral data presented above.

Closer examination of the K_d values for the [^{3}H]flunitrazepam binding analyses, without GABA, (Table 9a) reveals regional differences across cortex, cerebellum and hippocampus for all three strains. The highest BDZ receptor affinity for the control and resistant mice is seen in the cortex.

The BDZ receptor affinities for the cerebellum and hippocampus do not differ significantly. Similarly, the K_d values computed in the presence of GABA, do not show significant variation across brain regions. GABA is known to increase affinity of the BDZ receptor for agonists and this is evident in the data presented in Table 9b. Similar regional differences occur for the B_{max} values as well (Table 10a). While each strain illustrates some regional variation in the number of receptors present, this variation is not significant. This may be due, in part, to the relatively small sample size. These small regional differences in K_d and B_{max}, however, support the premise that BDZ binding is not a general membrane phenomena

In contrast to the subtle regional differences, distinct strain differences are also evident from these data. Significant differences in K_d (minus GABA) between the control (albino) and either drug resistant or drug sensitive mice in both the cortex and cerebellum are evident (Table 9a). While these data do not account for the behavioral differences between the resistant and sensitive strains, they do suggest genetically based dissimilarity in receptor affinity between the HS/IBg strain and the albino control strain. However, significant differences, in K_d ($p \leq .04$) do exist across the resistant and sensitive strains in the cerebellum (2.06 vs. 1.54 nM, respectively). It is interesting to note that the cerebellum is rich in type 1 BDZ receptors which have been classified as high affinity BDZ receptors (Klepner, 1979). Type 1 receptors have been hypothesized to mediate anxiolytic activity. It is possible to conclude that these results may be suggestive of a biochemical receptor link between the behaviorally distinct anxiolytic dose response profiles and the K_d values for this brain region. These data may be suggestive of the notion that the anxiolytic separation in dose response phenomena, identified in the behavioral studies, is mediated by a specific, significant difference in the cerebellar BDZ receptor affinity for the ligand. Replication of these data, with a much larger sample, is warrented before confirmation of a functional correlation between biochemical and behavioral responses could be made.

In the presence of GABA (Table 9b), the K_d for each strain is significantly decreased. This reflects what has been termed the "GABA shift" phenomena. GABA increases the affinity of the receptor for agonists which is reflected in a decrease in K_d. A three fold decrease in K_d is evident across all three brain regions. No significant change in the B_{max} values (Table 10b) are evident in the presence of GABA. These results are not unexpected since GABA does not affect the number of receptors present (B_{max}), but only the affinity (K_d) of BDZ receptor agonists such as flunitrazepam. There does seem to be a marginally significant difference between the Bmax values, in the presence of GABA, between the resistant and sensitive mice ($p \leq .08$). Judging from the small sample size (Table 8) in the hippocampus, any interpretation of this observation would be somewhat speculative. Further investigation in this region is warrented before any firm conclusions could be drawn.

Based on the data presented in tables 12-14 and figures 9 and 10, no significant difference exists between the drug resistant and drug sensitive mice in their response to diazepam potentiation of [^{35}S]TBPS binding. This study was undertaken in the hope of providing some insight into a possible genetic alteration in the interaction between GABA-gated chloride ion channel and the BDZ receptor. In this study, the effects of 10 concentrations of diazepam (0.00316-100.0 μM) were investigated.on the binding of 2 nM [^{35}S]TBPS. The average, linear and quadratic components of the resulting response profiles were analyzed (ALQ analysis). On the basis of these analyses, no statistically significant differences in the response profiles for both strains were found. It is important to note that variability in response for this study was very high (Table 11, Figures 9, 10) and sample sizes were low. One specific profile in the sensitive group was exceptionally high (Figure 8). Closer examination of the data reveals that the baseline "bound counts" for this replicate was low (158.0 cpm, as opposed to 376.3 to 620.3 cpm in the other replicates). Since the bound counts for all doses of diazepam were expressed as a percentage of this low baseline value, the

responses for this replicate were all high. Therefore, this single low baseline value generated the inconsistently high profile illustrated in Figure 9. This profile affected the statistical analyses to a considerable degree. If this replicate were to be deleted from the analysis, the p-value for strain difference in the average profile levels decreases to $p \leq .07$, indicating a trend towards significance

While the elimination of this one response profile is somewhat post-hoc, the net result suggests a marginal difference in the chloride ion channel interactions within the supramolecular complex. This information, when combined with the significant difference in the K_d value demonstrated in the cerebellum, suggests the need for further biochemical investigation. At present, these data have indicated the possibility of two different receptor interactions across two genetically selected strains of mice, in response to the effects of a known anxiolytic.

In order to confirm the statistical inferences which have been made, future replication of the biochemical profiles is warranted. The biochemical pharmacology presented here indicates that subtle difference in the BDZ receptor complex may exist between the DS and DR strains, in the cerebellum. One suggestion for future work is to expand the study in the hope of confirming these results and providing accurate estimation of the statistical significance of these receptor differences as they relate to the mediation of the behavioral differentiation evidenced by these studies.

SUMMARY AND CONCLUSIONS:

A novel behavioral model was established to test the anxiolytic effects of chlordiazepoxide in HS/IBg mice. Gallaher et al. (1987) developed two genetically divergent strains which may be functionally divided into two subgroups: resistant to the sedative effects of the anxiolytic diazepam (DR, drug resistant) and sensitive to the effects of diazepam (DS, drug sensitive). His data demostrated significant behavioral differences between the strains on their response to the sedative effects of Benzodiazepines. This new conflict model offers a major advantage over the older paradigms, i.e., a baseline is generated, using consummatory behavior as an operant, which is stable and permits each animal to serve as its' own control. This model is ideally suited for testing conflict behavior in small populations of animals

When the data were calculated as a percent of baseline, the DR mice were resistant toward the anxiolytic effects of chlordiazepoxide. A dose of 11.41 mg/kg was necessary to increase DR responding 100% over baseline while doses of 5.57 and 5.87 mg/kg induced the same percent increase in control and DS mice, respectively. These results suggest a genetic pre-selection for resistancy toward the anxiolytic properties of the benzodiazepines (BDZ). These data clearly do not support a difference in anxiolytic effects of BDZ between the sensitive and control mice. When baseline differences were ignored, the data clearly show a right shift in the dose response function for the DR mice. This major difference was most likely due to a decreased sensitivity to the sedative effects of chlordiazepoxide.

Behavioral data was also collected on the effects of chlordiazepoxide in potentiating the sedative effects (sleep time) of ethanol, across the DR and DS strains. The sleep times, induced

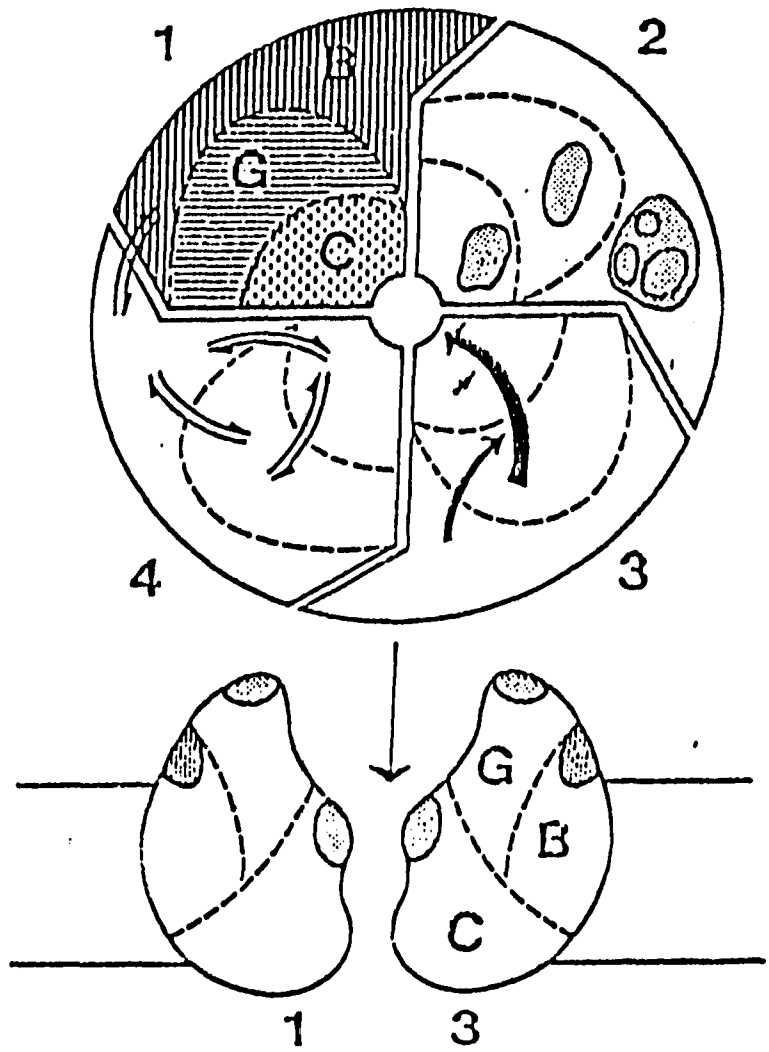
by 3 g/kg ethanol were not significantly different when compared across strains (DR = 45.1 min., DS = 45.5 min.) At 10 mg/kg chlordiazepoxide, the DR mice slept, a significantly shorter time (81.4 min.) than either the control (127.5 min.) or sensitive (121.6 min.) strain. Again, no differences in response were seen in the control and sensitive mice.

An examination of BDZ receptor pharmacology in the DR and DS strain was performed in order to correlate the significant behavioral selectivity identified by the behavioral profiles, to the anxiolytic effects of chlordiazepoxide, with possible neurobiochemical mediators. The DR and DS cortical tissue did not demonstrate significant variation in K_d or B_{max} values. The K_d values for the DR and DS strains were 0.96 and 0.95 nM, respectively. The B_{max} values for the DR and DS strains were 1579.6 and 1587.8 fmoles /mg protein. Significant differences were noted in the K_d values obtained in cerebellar tissue samples (DR = 2.06 nM, DS = 1.54 nM). While cortical, cerebellar and hippocampal samples all demonstrated increased affinity (K_d) in the presence of GABA, no significant differences were evident across strain. [^{35}S] TBPS binding was implemented and the results evaluated as a biochemical measure of chloride channel function (Skolnick et al., 1986). Statistical analysis (A-L-Q analysis) of cortical samples, across strain, indicated a significant difference between the DR (213.6 cpm) and DS (194.4 cpm) lines. Due to the small sample size and high variability, these results remain tentative pending further replication.

Based on these findings it is concluded that The DS and control mice, were not differentially sensitive to either the anxiolytic or sedative effects of chlordiazepoxide. The DR mice showed a shift to the right in their dose response effects to chlordiazepoxide. In addition the DR mice also evidenced decreased chlordiazepoxide potentiation of the sedative effects of ethanol. This differential response by the DR mice in response to

chlordiazepoxide can be accounted for by the reduced sedative effect of benzodiazepines in this strain. The biochemical profiles were for the most part unsuccessful in demonstrating a correlation betwen behavioral response and neurobiochemical mechanism of action. However, these biochemical data did provide leads both in significant K_d differences in cerebellar samples and [^{35}S] TBPS binding across the DR and DS lines. While small sample size generated high variability, trend differences were evident. These differences in biochemical responsivity will serve as starting points for future investigation and may generate a biochemical explaination for the genetic selection and subsequent behavioral response, in the DR and DS mice, toward the anxiolytic effects of the benzodiazepines.

Appendix 1



Hypothetical model of the benzodiazepine-GABA receptor-ionophore complex. The globular protein is seen from the extracellular space (top) and in a cross-section through the membrane (bottom). For explanation see text.

Appendix 2

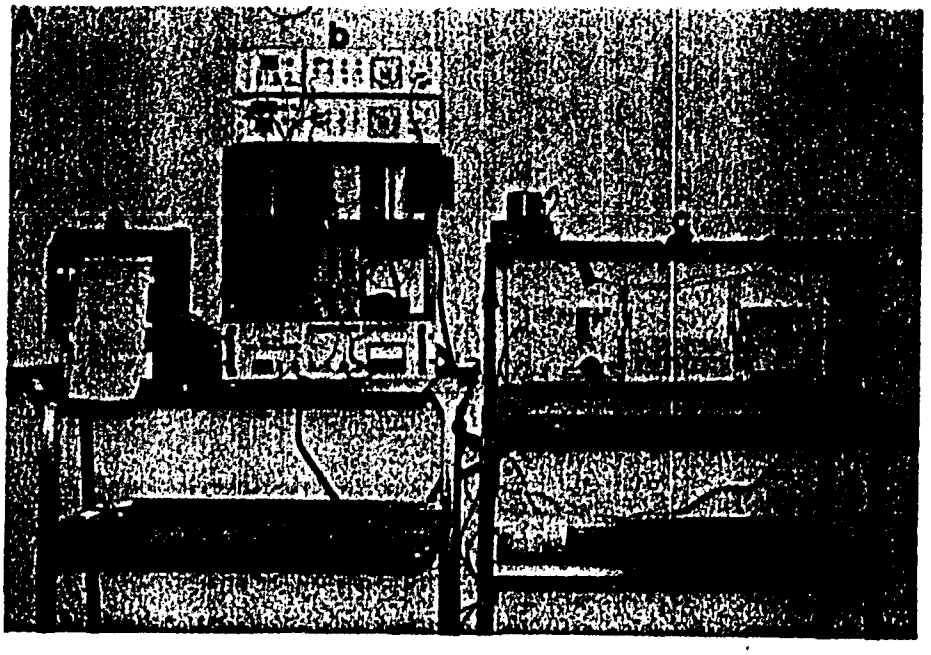


Figure A: (a) Cumulative Recorder
(b) Electro Mechanical Relay Equipment
(shock generator, timing devices - see methods)
(c) Test Apparatus (front panel open for viewing)

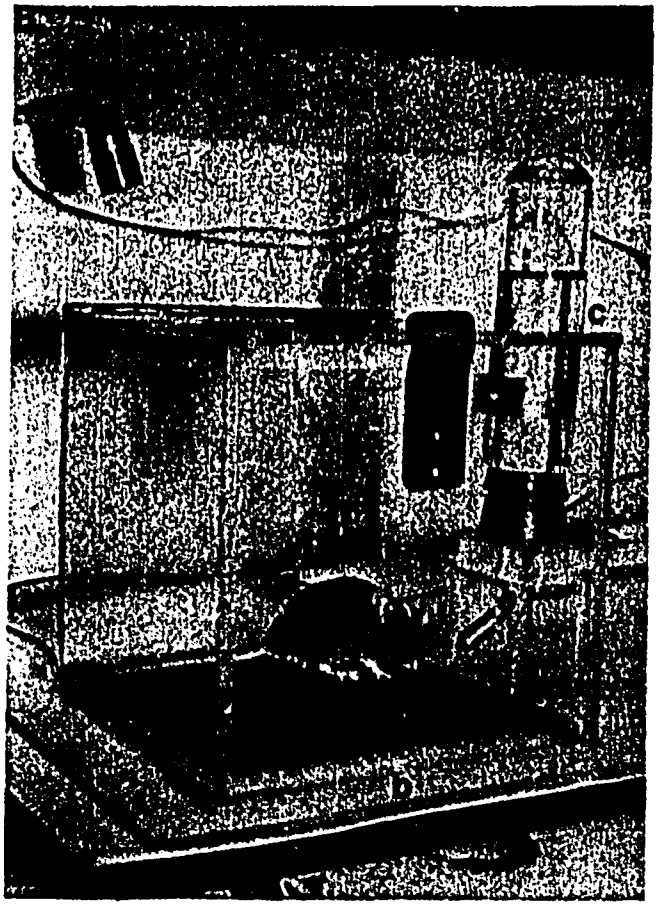


Figure B: (a) Light Source
(b) Grounded grid floor
(c) Electrified water tube
(d) Subject

BIBLIOGRAPHY

American Psychiatric Association. (1987) *Diagnostic and Statistical Manual of Mental disorders.* Third Edition. Washington, DC.

Babbini, M., Gaiardi, M. and Bartoletti, M. (1982) Benzodiazepine effects upon Geller-Seifter conflict tests in rats. *Pharmacol. Biochem. Behav.* **17,** 43-48.

Balter, M. B., Levine, J. and Manheimer, D. I. (1974) Cross-national study of the extent of anti-anxiety/sedative drug use. *N. Engl. J. Med.* **290,** 769-774.

Baraldi, M., Grandison, L. and Guidotti, A. (1979) Distribution and metabolism of muscimol in the brain and other tissues of the rat. *Neuropharmacology.* **18,** 57-62.

Bassett, J. R. and Cairncross, D. (1974) Effects of psychoactive drugs on response of the rat to aversive stimulation. *Arch. Int Pharmacodyn. Ther.* **212,** 221-229.

Beer, B., Chasin, M., Clody, D. E., Vogel, J. R. and Horovitz, Z. P. (1972) Cyclic adenosine monophosphate phosphodiesterase in brain. *Science* **176,** 428-430.

Beer, B. and Migler, B. (1975) Effects of diazepam on galvanic skin response and conflict in monkeys and humans. In *Predictability in Psychopharmacology: Preclinical and Clinical Correlations.* Sudilovsky, A., Gershon, S., and Beer, B., eds. Raven Press, New York, 143-157.

Benson, H., Herd, J. A., Morse, W. H. and Kellcher, R. T. (1970) Hypotensive effects of chlordiazepoxide, amobarbitol and chlorpromazine on behaviorally induced elevated arterial blood pressure in the squirrel monkey. *J. Pharmacol. Exp. Ther.* **173,** 399-406.

Berger, F. M. (1980) Effects of antianxiety drugs in fear and stress. *Behav. Sci.* **25,** 315-321.

Billingsley, M. L. and Kubena R. K. (1978) The effects of naloxone and picrotoxin on the sedative and anticonflict effects of benzodiazepines. *Life Sci.* **22,** 897-906.

Birk, J. and Noble, R. G. (1981) Naloxone antagonism of diazepam-induced feeding in the syrian hamster. *Life Sci.* **30,** 321-325.

Bonetti, E. P., Pieri, L., Cumin, R., Schaffner, R., Gamzu, E. R., Muller, R. M. and Haefley, W. (1982) Benzodiazepine antagonist Ro 15-1788: neurological and behavioral effects: *Psychopharmacology* **78,** 8-18.

Brady, J. V. and Hunt H. F. (1955) An experimental approach to the analysis of emotional behavior. *J. Psychol.* **40,** 313-324.

Braestrup, C. and Nielsen, M. (1979) Partial agonist for brain GABA/ benzodiazepine receptor complex. *Nature* **280,** 331-333.

Braestrup, C. and Nielsen, M. (1980) Modulation of the benzodiazepine receptors. *Adv. Biosci.* **31,** 221-227.

Braestrup, C. and Nielsen, M. (1982) Anxiety. *Lancet* **2(8306),** 1030-34.

Braestrup, C. and Nielsen, M. (1983) In *Handbook of Pharmacology* Iversen, L. L., Iversen, S. D. and Snyder, S. H. eds. **17,** 285-384.

Braestrup, C., Nielsen, M. and Olsen, C. E. (1980) Urinary and brain beta-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. Natl. Acad. Sci. USA* **77(4),** 2288-92.

Brown, R. K., Houpt, K. A. and Schryver, H. F. (1976) Stimulation of food intake in horses by diazepam and promazine. *Pharmacol. Biochem. Behav. 5,* 495-497.

Carlton, P. L. (1978) Theories and models in psychopharmacology. In: *Psychopharmacology: A Generation of Progress.* Lipton, M. A., DiMascio, A. and Killam, K. F. eds. New York: Raven Press.

Chang, R. S. L. and Synder, S. H. (1978) Benzodiazepine receptors: labeling intact animals with [3H]flunitrazepam. *Eur. J. Pharmacol.* **48,** 213-218.

Childress, S. J. and Gluckman M. I. (1964) 1,4 Benzodiazepines. *J. Pharm. Sci.* **53,** 577-590.

Christmas, A. J. and Maxwell, D. R. (1970) A comparison of the effects of some benzodiazepines and other drugs on aggressive and exploratory behavior in mice and rats. *Neuropharmacology* **9,** 17-29.

Clody, D. E., Lippa, A. S. and Beer, B. (1982) Preclinical procedures as predictors of antianxiety activity in man. In: *Pharmacology of Benzodiazepines.* Usdin, S., Skolnick, P., Tallman, J. and Greenblatt, D. eds. London: Macmillan Press.

Cook, L. and Davidson, A. B. (1973) Effects of behaviorally active drugs in a conflict-punishment procedure in rats. In: *The Benzodiazepines.* Garattini, S., Mussini, E. and Randall, L. O. eds. New York: Raven Press.

Cook, L. and Sepinwall, J. (1975) Behavioral analysis of the effects and mechanisms of action of benzodiazepines. In: *Mechanisms of Action of Benzodiazepines.* Costa, E. and Greengard, P. eds. New York: Raven Press.

Cooper, S. J. and Francis R. L. (1979) Effects of acute or chronic administration of chlordiazepoxide on feeding parameters using two food textures in the rat. *J. Pharm. Pharmacol.* **31,** 743-746.

Cooper, S. J. and Posadas-Andrews, A. (1979) Food and water intake in the non-deprived pigeon after chlordiazepoxide administration. *Psychopharmacology* **65,** 99-101.

Costa, E. and Greengard, P. (1978) *Mechanisms of Action of Benzodiazepines.* New York: Raven Press.

Crawley, J. N. and Goodwin, F. K. (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol. Biochem. Behav.* **13,** 167-170.

Dantzer, R. (1978) Antipunishment effects of diazepam: Interaction with shock and food deprivation levels in pigs. *Psychopharmacology* **58,** 99-104.

Darwin, C. (1963) On the origins of species by means of natural selection: or the preservation of favored races in the struggle for life. New York: Heritage Press.

Delini-Stula, A. (1971) Drug induced suppression of conditioned hyperthermic and conditioned avoidance behavior response in rats. *Psychopharmacologia* **20,** 153-159.

Della-Fera, M. A., Baile, C. A. and McLaughlin, C. L. (1980) Feeding elicited by benzodiazepine-like chemicals in puppies and cats. *Pharmacol. Biochem. Behav.* **12,** 195-200.

Dunham, N. W. and Miya, T. A. (1957) A note on a simple apparatus for detecting neurological deficit in rats and mice. *J. Amer. Pharmac. Assoc.* **46,** 208-209.

Estes, W. K. and Skinner, B. F. (1941) Some quantitative properties of anxiety. *J. Exp. Psychol.* **29,** 390-400.

Fabre, L. F. and McLendon, D. M. (1980) A double-blind study comparing the efficacy and safety of alprazolam with imipramine and placebo in primary depression. *Curr. Ther. Res.* **27,** 474-482.

Fantino, E. (1973) Aversive control. In: *The Study of Behavior, Learning Motivation, Emotion and Instinct.* Nevin, J. A. and Reynolds, G. S. eds. Glenview, IL. Scott, Foresman.

File, S. E. (1982) The rat corticosterone response: habituation and modification by chlordiazepoxide. *Physiol. Behav.* **29,** 91-95.

File, S. E. (1986) Animal models for predicting clinical efficacy of anxiolytic drugs: social behavior. *Neuropsychobiology* **13,** 55-62.

File, S. E., Greenblatt, D. J., Martin, I. L., and Brown, C. (1985) Long-lasting anticonvulsant effects of diazepam in different mouse strains: Correlations with brain concentrations and receptor occupancy. *Psychopharmacology* **84,** 137-146.

File, S. E. and Hyde J. R. G. (1978) Can social interaction be used to measure anxiety? *Br. J. Pharmac.* **62,** 19-24.

File, S. E. and Hyde J. R. G. (1979) A test of anxiety that distinguishes between the actions of benzodiazepines and those of other minor tranquilizers and of stimulants. *Pharm. Biochem. Behav.* **11,** 115-122.

File, S. E. and Pellow, S. (1984) The anxiogenic action of Ro 15-1788 is reversed by chronic, but not acute, treatment with chlordiazepoxide. *Brain Res.* **310,** 154-156.

File, S. E. and Pellow, S. (1985) The benzodiazepine receptor antagonist Ro 15-1788 has an anxiogenic action in four animal tests of anxiety. *Br. J. Pharmac.* **84,** 103-105.

Gallaher, E. J., Parsons, L. M. and Goldstein, D. B. (1982) The rapid onset of tolerance to ataxic effects of ethanol in mice. *Psychopharmacology* **78,** 67-70.

Gallaher, E. J. and Gionet, S. E. (1986) Initial sensitivity and tolerance to ethanol in mice genetically selected for diazepam sensitivity. *Clin. Exper. Res.* **52,** 256-311.

Garcia, J. and Koelling, R. (1973) Relation of cue to consequence in avoidance learning. *Psychon. Sci.* **4,** 123-124.

Garrett, K. M. and Tabakoff, B. (1985) The development of type I and type II receptors in the mouse cortex and cerebellum. *Pharmacol. Biochem. Beh.* **22,** 985-992.

Geller, I. and Blume K. (1970) The effects of 5-HTP on parachlorophenylalanine (p-cPA) attenuation of "conflict" behavior. *Eur. J. Pharmacol.* **9,** 319-324.

Geller, I. and Seifter, J. (1960) The effects of meprobamate, barbiturates, D-amphetamines and promazine on experimentally induced conflict in the rat. *Psychopharmacologia* **1,** 482-492.

Glick, S. D. (1976) Screening and therapeutics, animal models and human problems. In: *Behavioral Pharmacology* Glick, S. D. and Goldfarb, J. eds. St. Louis: Mosby.

Goodman, L. S. and Gilman, A. (1980) *The Pharmacological Basis of Therapeutics,* New York: Macmillan.

Greenblatt, D. J. and Shader, R. I. (1978) Dependence, tolerance and addiction to benzodiazepines: Clinical and pharmacokinetic considerations. *Drug Metab. Res.* **8,** 13-28.

Guidotti, A., Baraldi, M., Leon, A. and Costa, E. (1980) Benzodiazepines: A tool to explore the biochemical and neurophysiological basis of anxiety. *Federation Proceedings* **39,** 3039-3042.

Haefely, W., Polc, P., Schaffner, R., Keller, H., Pieri, L. and Mohler, H. (1979) Facilitation of GABAergic transmission by drugs. In: *GABA-Neurotransmitters.* Krogsgaard-Larsen, P., Scheel-Kruger, J. and Kofod, M. eds. Copenhagen: Munksgaard, 357-375.

Haefely, Kyburz, E., Gerecke, M and Mohler, H. (1985) Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationships of their agonists and antagonists. *Adv. in Drug Res.* **14,** 165-222.

Havoundjian, H., Paul, S. M. and Skolnick, P. (1986) The permeability of aminobutyric acid-gated chloride channels is described by the binding of a "cage" convulsant, t-butylbicyclophsophoro[^{35}S]-thionate. *Proc. Natl. Acad. Sci. USA* **83,** 9241-9244.

Henriksson, B G. and Jarbe, T. (1971) Effects of diazepam on conditioned avoidance learning in rats and its transfer to normal state conditions. *Psychopharmacologia* **20,** 186-190.

Hill, R. T. and Tedeschi, D. H. (1971) Animal testing and screening procedures in evaluating psychotropic drugs. In: *An introduction to pharmacology.* Rech, R. H. and Moore, K. E. eds. New York: Raven Press.

Hunkeler, W., Mohler, H., Pieri, L., Polo, P., Bonetti, E. P. Cumin R., Schaffner, R., and Haefely H. (1981) Selective antagonists of benzodiazepines. *Nature* **290,** 514-516.

Irwin, S. (1967) Anti-neurotics: Practical pharmacology of sedative-hypnotics and minor tranquilizers. In: *Pharmacology: A Review in Progress.* Efron, D. H. eds. U.S. public Health Service Publication No. 1836.

Itoh, H. and Takaori, S. (1968) Effects of psychotropic agents on the exploratory behavior of rats in a Y-shaped box. *Jpn. J. Pharmacol.* **18,** 344-352.

Iwahara, S. and Sakama, E. (1972) Effects of chlordiazepoxide upon habituation of open-field behavior in white rats. *Psychopharmacologia* **27,** 285-292.

Jacobsen, E. (1957) The effects of psychotropic drugs under psychic stress. In: *Psychotropic Drugs.* Garattini, S. and Ghetti, V. eds. Amsterdam: Elsevier.

Johnston, G. A. R. and Willow, M. (1982) GABA and barbiturate receptors. *Trends Pharm. Sci.* **3,** 328-330.

Karobath, M. and Sperk, G. (1979) Stimulation of benzodiazepine receptor binding by gamma-amino butyric acid. *Proc. Natl. Acad. Sci.* **76,** 1004-1006.

Karobath, M., Drexler, G. and Supavila, P. (1981) Modulation by picrotoxin and IPTBO of [^{3}H]flunitrazepam binding to the GABA-benzodiazepine receptor complex of rat cerebellum. *Life Sci.* **28,** 307-313.

Keim, K. L. and Sigg, E. B. (1977) Plasma corticosterone and brain catecholamines in stress: Effects of psychotropic drugs. *Pharmacol. Biochem. Behav.* **6,** 79-85.

Kelly, D. (1980) Beta-blockers in anxiety. *J. Pharmacother.* **1**, 91-98.

Klepner, C. A., Lippa, A. S., Benson, D. I., Sano, M. C. and Beer, B. (1979) Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharacol. Biochem. Behav.* **11**, 457-462.

Kumar, R. (1971) Effects of chlordiazepoxide and chlorpromazine on fear and exploratory behavior in rats. *Psychopharmacologia* **19**, 297-312.

Kuribara, H. and Tadokoro, S. (1979) Effects of psychotropic drugs on avoidance response in rats: Role of baseline performances. *Pharmacol. Biochem. Behav.* **11**, 595-603.

Langley, J. N. (1905) On the reaction of cells and nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curare. *J. Physiol.* (London) **33**, 374-413.

Laurian, S., Gaillard J. M. and Schopf J. (1984) Effects of a benzodiazepine antagonist on the diazepam-induced electrical brain activity modifications. *Neuropsychobiology* **11**, 55-58.

Lefur, G., Guilloux, F., Mitrani, N., Mizoule, J. and Uzan, A. (1979) Relationships between plasma corticosteroids and benzodiazepines in stress. *J. Pharmacol. Exp. Ther.* **211**, 305-308.

Leeb-Lundberg, F., Snowman, A. and Olsen, R. W. (1980) Barbiturate receptor sites are coupled to benzodiazepine receptors. *Proc. Natl. Acad. Sci. USA.* **77**, 7468-7472.

Liljequist, S. and Engel J. A. (1984) Reversal of the anti-conflict action of valproate by various GABA and benzodiazepine antagonists. *Life Sci.* **34**, 2525-2533.

Liljequist, S., Garrett, K. M. and Tabakoff B. (1984) Specific effects of chronic phenobarbitol administration on high and low-affinity benzodiazepine receptors in mouse cerebellum and forebrain. *Eur. J. Pharmacol.* **103,** 343-348.

Lippa, A. S., Klepner, C. A., Yunger, L., Sano, M. C., Smith, W. V., and Beer, B. (1978) Relationship between benzodiazepine receptor and experimental anxiety in rats. *Pharmacol. Biochem. Behav.* **9,** 853-856.

Lippa, A. S., Critchett, D., Sano, M. C., Klepner, C. A., Greenblatt, E. N., Coupet, J., and Beer, B. (1979) Benzodiazepine receptors: cellular and behavioral characteristics. *Pharmacol. Biochem. Behav.* **10,** 831-843.

Lowry, O.H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Margules, D. L. and Stein, L. (1968) Increase of antianxiety activity and tolerance of behavioral depression during chronic administration of oxazepam. *Psychopharmacologia* **13,** 74-80.

Marriott, A. S. and Smith, E. F. (1972) An analysis of drug effects in mice exposed to a simple novel environment. *Psychopharmacologia* **24,** 397-406.

Methot, C. and Deutsch, R. (1984) The effects of diazepam on a conditioned emotional response in rats. *Pharmacol. Biochem. Behav.* **20,** 459-499.

Morrison, D. F. (1976) *Multivariate Statistical Methods.* **4,** New York: McGraw Hill.

Nutt, D. J., Cowen, P. J. and Little, H. J. (1982) Unusual interactions of benzodiazepine receptor antagonists. *Nature* **295,** 436-438.

Patel, J. B. and Migler, B. (1982) A sensitive and selective monkey conflict test. *Pharmacol. Biochem. Behav.* **17,** 645-649.

Petersen, E. N. and Lassen, J. B. (1981) A water lick conflict paradigm using drug experienced rats. *Psychopharmacology (Berlin)* **75,** 236-239.

Pollard, G. T. and Howard, J. L. (1979) The Geller-Seifter conflict paradigm with incremental shock. *Psychopharmacology* **62,** 117-121.

Ramanjaneyulu, R. and Ticku, M. K. (1984) Binding characteristics and interaction with depressant drugs with [^{35}S] t-butyl-bicyclophosphorothianate, a ligand that binds to the picrotoxin site. *J. Neurochem.* **42,** 221-229.

Randall, L. O. and Kappell, B. (1961) Pharmacology of chlordiazepoxide (librium) and analogues. *Biochem. Pharmacol.* **8,** 15-17.

Rommelspacher, H. (1981) The B-carbolines (tharmanes) a new class of endogenous compounds: their relevance for the pathogenesis and treatment of psychiatric and neurological diseases. *Pharmacopsychiatry* **14,** 117-125.

Sansone, M. (1978) Effects of chlordiazepoxide, amitriptyline, imipramine and their combinations on avoidance behavior in mice. *Psychopharmacology* **59,** 151-155.

Scobie, S. R. and Garske, G. (1970) Chlordiazepoxide and conditioned suppression. *Psychopharmacologia* **16,** 272-280.

Sepinwall, J. and Cook, L. (1978) Behavioral pharmacology of anti-anxiety drugs. In: *Psychopharmacology.* Iverson, S. D. ed. New York: Raven Press.

Skolnick, P., Moncada, V., Barker, J. L. and Paul, S. M. (1981) Pentobarbitol has dual actions to increase brain benzodiazepine receptor affinity. *Science* **211,** 1448-1450.

Skolnick, P., Schweri, M. M., Williams, E. F., Moncada, V. Y., and Paul, S. M. (1982) An in vitro binding assay which differentiates benzodiazepine "agonists" and "antagonists." *Eur. J. Pharmacol.* **78,** 133-136.

Snedecor, G. and Cochran, W. (1980) *Statistical Methods.* Iowa State University Press.

Snell, D. and Harris, R. A. (1980) Impairment of avoidance behavior following short-term ingestion of ethanol, tertiary-butanol and pentobarbitol in mice. *Psychopharmacology* **69,** 53-37.

Soubrie, P., DeAngelis, L., Simon, P. and Boissier, J. R. (1976) Effects des anxiolytiques sur la prise de boisson en situation nouvelle et familiere. *Psychopharmacologia* **50,** 41-45.

Sperk, G. and Schloegl, E., (1979) Reduction of number of benzodiazepine binding sites in the caudate nucleus of the rat after kianic acid injections. *Brain Res.* **170,** 563-567.

Squires, R. F., Benson, D. I., Braestrup, C. Coupet, J., Klepner, C. A., Myers, V. and Beer, B. (1979) Some properties of brain-specific benzodiazepine receptors: new evidence for multiple receptors. *Pharmacol. Biochem. Behav.* **10,** 825-830.

Stein, L., Wise, C. D., and Belluzzi, J. D., (1975) Effects of benzodiazepines on central serotonergic mechanisms. In: *Mechanism of Action of Benzodiazepines.* Costa, E. and Greengard, P. eds. New York: Raven Press.

Stein, L. and Berger, B.O. (1969) Paradoxical fear-increasing effects of tranquilizers: Evidence of repression of memory in the rat. *Science* **166,** 253-256.

Stephenson, R. P. (1956) A modification of receptor theory. *Br. J. Pharmacol.* **11,** 379-393.

Tallman, T. F., Paul, M., Skolnick, P. and Gallager, D. W. (1980) Receptors for the age of anxiety. Pharmacology of the benzodiazepines. *Science* **207,** 274-281.

Ticku, M. K. Interaction of depressant, convulsant and anticonvulsant barbiturates with [^{3}H] diazepam bindings sites at the benzodiazepine-GABA receptor ionophore complex. *Biochem. Pharmacol.* **30,** 1573-1579.

Tobin, J. M. and Lewis, N.D. (1960) New psychotherapeutic agent, chlordiazepoxide. Use in treatment of anxiety states and related syndroms. *JAMA.* **174,** 1242-1249.

Tukey, J. W., Ciminera, J. L. and Heyse, J. F. (1985) Testing the statistical certainty of a response to increasing doses of a drug. *Biometrics* **41,** 295-301.

Vogel, J. R., Beer, B. and Clody, D. E. (1971) A simple and reliable conflict procedure for testing anti-anxiety agents. *Psychopharmacologia* **21,** 1-7.

Williamson, M. J., Paul, S. M. and Skolnick, P. (1978) Demonstration of [^{3}H]diazepam binding to benzodiazepines receptors *in vivo*. *Life Sci.* **23,** 1935-1940.

Witkin, J. M. and Barrett, J. E. (1981) Effects of pentobarbitol on punished behavior: Persistent increases with chronic administration. *Psychopharmacology (Berlin)* **75,** 324-325.

Yates, F. (1982) Regression models for repeated measurements. *Biometrics* **38,** 830-833.

Young, A. B., Zukin, S. R. and Snyder, S.H. (1974) Interactions of benzodiazepines with central nervous system receptors: possible mechanism of action. *Proc. Natl. Acad. Sci. U.S.A.* **71,** 2246-2250.