A PSYCHOPHARMACOLOGICAL STUDY OF ANXIETY IN MICE AND PIGS

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This thesis is my own account of investigations carried out predominantly by myself under the supervision of Dr. J.P. Fry.

Abstract

An attempt was made to study the psychological state of anxiety through the development and evaluation of two behavioural tests; an exploratory conflict test in the mouse and a pharmacological conditioning procedure in the pig. The tests were used to investigate hormonal and environmental influences on the anxiety state of these animals.

The conflict arising from a light/dark choice exploratory situation was examined in male mice for its sensitivity to the anxiolytic 1,4-benzodiazepine diazepam. Only one parameter of behaviour in this test (light/dark transitions) showed a dose related increase to diazepam in male mice. Further experiments revealed that a diazepam induced increase in this parameter of behaviour does not reflect a specific anti-anxiety drug action. Experiments which indicate that the light/dark choice test does not detect an anxiolytic action of diazepam are described. The parameter of light/dark transitions did not fluctuate in untreated female mice during the oestrous cycle. However, a diazepam induced change similar to that observed in males was found only at oestrus and dioestrus. Mice at pro-oestrus, metoestrus II and late dioestrus did not show this response to diazepam while mice at metoestrus I showed a diazepam induced decrease in the number of light/dark transitions. This effect of diazepam at metoestrus I was paralleled by changes in other test parameters indicative of an anxiogenic drug action or a photophobic effect. Measurements of whole brain concentrations of diazepam revealed that these behavioural fluctuations in response to diazepam during the oestrous cycle are not due to a change in drug metabolism or distribution.

In the pharmacological conditioning experiments, pigs were trained to discriminate the anxiogenic drug pentylenetetrazole from an injection of the saline vehicle alone. A conditioning procedure was developed whereby the time course of the pentylenetetrazole cue could be measured. The behavioural and pharmacological specificity of this cue was investigated. This study revealed that the cue corresponds to an anxiety state. Application of this technique to a range of environmental stimuli which mimic conditions encountered in pig husbandry revealed its ability to detect the presence and time course of anxiety in these situations.

Abbreviations

ACTH	adrenocorticotropic hormone
ß-CCE	ß-carboline-3-carboxylate ethyl ester
ß-CCM	ß-carboline-3-carboxylate methyl ester
ß-CCP	ß-carboline-3-carboxylate propyl ester
BSA	bovine serum albumin
CER	conditioned emotional response
CES	conditioned emotional state
CGS8216	2-phenyl pyrazolo[4,3-C]quinolin-3(5H)-one
CL218872	6[(3-Trifluoromethyl)phenyl]-3-methyl-1,2,4-triazolo[4,3-6]pyridazine
CNS	central nervous system
CRF	corticotropic releasing factor
CS	conditioned stimulus
DMCM	methyl-6,7-dimethyl-4-ethyl-ß-carboline-3-carboxylate
dpm	disintegrations per minute
ED_{50}	dose of drug which induces a response in 50% of the subjects tested
FG7142	ß-carboline-3-carboxylic acid methyl amide
FR	fixed ratio
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
[3H]	tritium
5HT	5-hydroxytryptamine
IC_{50}	concentration of displacer which inhibits 50% of control binding
i.p.	intraperitoneal
i.v.	intravenous
KD	kilodalton
MW	molecular weight
NA	noradrenaline
no.	number
3α-OH-DHP	3α-hydroxy 5α-pregnan-20-one
PCPA	parachlorophenylalanine
p.o.	per os
PTZ	pentylenetetrazole
s.c.	subcutaneous
S.E.M.	standard error of the mean
TBPS	t-butylbicyclophosphorothionate
THDOC	3α,21-dihydroxy-5α-pregnan-20-one
UCS	unconditioned stimulus
VI	variable interval
VR	variable ratio
ZK91296	5-benzyloxy-4-methoxymethyl-ß-carboline-3-carboxylic-acid-ethyl-ester
ZK93423	6-benzyloxy-4-methoxymethyl-ß-carboline-3-carboxylate ethyl ester
ZK93426	5-isopropoxy-4-methyl-ß-carboline-3-carboxylic acid ethyl ester

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Chapter 1

Introduction

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1.1 Introduction

Anxiety has been defined as an emotional state resembling fear, provoked by anticipation of an unpleasant stimulus (Estes & Skinner, 1941). This psychological state is commonly experienced by man and may serve a useful purpose by inducing arousal. However, anxiety becomes abnormal when it occurs without objective reasons or when its manifestations are severe in intensity and duration. Clinical anxiety disorders are amongst the most frequently encountered psychopathological conditions (Lader, 1986). An understanding of the neurological mechanisms underlying anxiety has been facilitated in recent years by the availability of drugs with which the condition can be modified and by an elucidation of the mechanism of action of these drugs.

The 1,4-benzodiazepine class of minor tranquillising drugs, e.g. chlordiazepoxide (librium) and diazepam (valium), are currently the most commonly prescribed anti-anxiety agents. These drugs have other therapeutic applications as anticonvulsants, muscle relaxants and hypnotics (Zbinden & Randall, 1967; Randall et al., 1974). The pharmacological actions of the 1,4-benzodiazepines are mediated by specific receptor binding sites in the vertebrate central nervous system (CNS) (Möhler & Okada, 1977; Squires & Braestrup, 1977) which are coupled to receptors for the major inhibitory neurotransmitter γ-aminobutyric acid (GABA; see Olsen, 1982). While the benzodiazepine tranquillisers have proved to be valuable tools in providing insight into the neurobiology of anxiety, studies of anxiety have been further advanced by the discovery of agents which act through the GABA/benzodiazepine receptor system but exert pharmacological actions opposite to those of the 1,4-benzodiazepines (Polc et al., 1982; Braestrup et al., 1982 and see Braestrup et al., 1984). In contrast to the anxiety alleviating (anxiolytic) properties of the classical benzodiazepines, these agents act to provoke anxiety (anxiogenic).

Although clinical trials have provided valuable information on the subjective effects of treatments with such drugs, animal models are essential

to study both the mechanisms by which anxiolytic or anxiogenic drugs modulate anxiety and the neurobiology of this state.

In the present chapter, several of the available animal models of anxiety are described, the information which has accumulated on the structure and function of the GABA/benzodiazepine receptor system and the characteristics of the benzodiazepine ligands is reviewed and finally, the validity of animal tests as models of anxiety is discussed.

1.2 Animal models of anxiety

Since an assessment of anxiety in humans largely consists of subjective verbal reports, the development of animal analogues of this psychological state has not been an easy task. In the development of animal tests of anxiety, attempts have been made to reproduce aspects of human anxiety in animals. For the tests described in this section, such aspects include conflict and the anticipation of an unpleasant stimulus.

Conflict situations are reflected by disruption of the ongoing behaviour of an animal induced by presentation of an aversive stimulus. Some of the animal tests described here employ conditioned conflict. These tests control the behaviour of the animal through various operant food or water reinforcement schedules and conflict is created when such reinforcements are coupled with punishment. Other tests of conflict make use of the natural exploratory tendencies of rodents and in these models conflict is represented by a disruption of the ongoing exploratory behaviour of the animal, induced by the presentation of aversive stimuli or neutral stimuli conditioned aversively.

1.2.a Models based on punishment-induced conflict

The Geller-Seifter conflict test

The Geller-Seifter conflict test was first developed in 1960 and since then it has been widely adopted and modified by other workers. The original test procedure as described by Geller and Seifter (1960) is as follows: Food

restricted rats are trained to respond for food reinforcement in an operant chamber on two schedules of reinforcement. One schedule is a variable interval (VI) schedule of 2 mins whereby reinforcement is delivered after a mean interval of 2 mins. The other schedule is a continuous reinforcement schedule (CRF) where each lever press results in food reward. The transition from the VI schedule to the CRF schedule is signalled by an auditory stimulus. This stimulus remains for the duration of the CRF period. Once stable responding has been obtained on both schedules, each reinforcement in the CRF period is paired with electric shock. Thus during the signalled CRF period a conflict is created whereby 'the animals must balance the positive features of a higher reward payoff against the negative aspects of accepting pain shocks' (Geller & Seifter, 1960).

Typically, a suppression of response occurs during the punished CRF period. Measurement of response rate in the unpunished schedule in the same session allows the separation of treatments with true anti or pro-conflict activity from those which cause a general increase or decrease in motor activity. A reduction in conflict is reflected by an increase in the rate of response during the CRF/shock period. Treatments which induce this behaviour without affecting the VI schedule of response are interpreted as anxiolytic. By manipulating the level of shock and therefore the baseline level of response during the CRF/shock period it is possible to detect further suppression of responding. Treatments which produce this result without affecting the control schedule are interpreted as anxiogenic.

This test has been modified for use in mice whereby an unsignalled punished period is preceded and followed by unpunished periods of the same duration and on the same reinforcement schedule (De Carvalho et al., 1983).

The Vogel drink conflict test

The model of Vogel et al. (1971) is based on the conflict created by punishing drinking behaviour in water deprived rats. The index of anxiety measured is the number of licks the animal makes in a session where typically each 20th lick made is punished by electric shock. Treatments which attenuate

the reduction of licking behaviour seen in untreated animals are considered to be anxiolytic. Modifications of the original procedure described by Vogel et al. (1971) include the titration of shock level in order to detect further suppression of punished licking induced by putative anxiogenic treatments. To control for the possible influence of drugs on motivational or motor capability of licking, a predrug punished session or a postdrug unpunished session can be included in the protocol (Petersen et al., 1983; Stephens et al., 1987).

The four plate test

The four plate test, first employed by Slotnik and Jarvik (1966) to study the effects of septal lesions on passive avoidance and conditioned fear, represents an approach avoidance conflict whereby the natural tendency of mice to explore a novel environment is negatively reinforced by punishment (Boissier et al., 1968). The apparatus consists of a rectangular box with a floor of four metal plates separated from each other by a gap of 4 mm. When the mouse crosses from one plate to another it receives an electric shock. The index of anxiety measured is the number of punished crossings. Treatments which attenuate the suppression of punished crossings seen in untreated mice are considered anxiolytic. Anxiogenic treatments are expected to cause a further enhancement in the reduction of punished crossings when the shock level is titrated to overcome the floor effect of punished suppressed behaviour seen in untreated animals. As a control for non-specific locomotor effects of drug treatments, an assessment can be made of the effects of such drugs on non-punished crossings in independent groups of mice (Stephens & Kehr, 1985).

1.2.b Models based on exploratory behaviour

The Holeboard test

The holeboard apparatus was first introduced by Boissier and Simon (1962) as a test of exploration in the mouse, on the assumption that curiosity and fear aroused by a novel stimulus would give rise to exploratory and escape behaviour. The original apparatus consisted of a square board with sixteen equally spaced holes. The index of exploration measured was the number of head dips into the holes. Subsequently this apparatus was modified for use in

rats and mice and the number of holes were reduced to four in an attempt to distinguish general motor activity from true exploratory activity (File & Wardill, 1975). The index of exploration measured in the four holeboard test is the number of head-dips and the time spent head-dipping. Locomotor activity in the apparatus is also measured to ensure that an increase or decrease in the index of exploration is not due to a general shift in the level of motor activity of the animal.

The light/dark choice test of exploration

The light/dark choice test of exploration exploits the natural exploratory tendency of mice and their preference for dark places. The proposed conflict is the tendency of mice to explore a brightly lit chamber versus the tendency to retreat into a dark chamber (Crawley & Goodwin, 1980). The exploratory apparatus consists of two chambers separated by a tunnel. One chamber is brightly lit and twice the size of the other chamber which is darkened. The index of exploration measured is the number of light/dark tunnel transitions; an increase in this parameter reflecting an anxiolytic action and a decrease an anxiogenic action. A non-specific action of a drug treatment on locomotory behaviour is distinguished from an anxiolytic or anxiogenic action by assessing the effects of the same drug on motor performance in a bare undifferentiated box (Crawley & Goodwin, 1980). Further details and a characterisation of this test are provided in Chapters 2 and 4.

The social interaction test

When pairs of male rats are placed on neutral territory they perform a variety of behavioural actions towards each other, including exploratory sniffing and grooming. The baseline level of such social interaction can be varied as a function of the familiarity and illumination intensity of the test arena. Animals have higher social interaction scores when tested in a familiar low light arena than in a bright unfamiliar arena. The decrease in social behaviour displayed in the latter test condition is thought to reflect the uncertainty of the environment. On the basis of the above findings the social interactive behaviour of male rats under the two variable test conditions, has

been proposed as an animal analogue of anxiety (File & Hyde, 1978). The index of anxiety employed is the time spent by pairs of male rats in active social interaction. Treatments which increase social interaction in the bright unfamiliar arena to the level seen in the familiar low light arena are interpreted as anxiolytic while treatments which produce the converse are taken to be anxiogenic. General locomotor activity around the test arena and the amount of passive social interaction are distinguished from active interaction and are not included in the anxiety score.

The social interaction test has been modified for use with mice whereby the familiarity parameter is not varied since social interaction in mice was found not to change as a function of the degree of familiarity of the test arena (De Angelis & File, 1979).

The elevated plus maze

The elevated plus maze test is based on the natural aversion of rodents for open elevated spaces as reported by Montgomery (1955), who demonstrated a greater approach avoidance conflict in rats when exposed to an elevated open maze than to an enclosed maze alley. The elevated plus maze apparatus consists of four wooden arms at right angles to each other, connected to a central square. The apparatus is raised from the ground; 50-70 cm for rats and 38.5 cm for mice. Two of the opposite arms have high wooden walls (enclosed arms) whereas the other two do not (open arms). The central square is not enclosed and the rodent is initially placed in this area facing an enclosed arm. Two indices of anxiety are measured, the number of entries into the open arms expressed as a proportion of the total number of arm entries and the amount of time spent on the open arms expressed as a proportion of the total time on both open and closed arms. An anxiolytic effect is reflected by an increase in both of these indices while a decrease in these indices is interpreted as an anxiogenic effect (Handley & Mithani, 1984; Pellow et al., 1985; Lister, 1987b).

1.2.c The Conditioned Emotional Response (CER) paradigm

The CER paradigm was first proposed by Estes & Skinner (1941) as a behavioural prototype of anxiety. The procedure used by these authors and subsequently adopted and modified by many others is as follows: food deprived rats were trained to respond on a lever in an operant chamber for food reinforcement which was delivered after fixed intervals of 4 minutes. Once responding was reliably established on this schedule, a tone stimulus was sounded for three or five minutes and was terminated by an electric shock applied to the feet. Initially the tone or shock did not affect operant responding. However, on repeated presentation of tone shock pairings, a progressive suppression of response occurred during the preshock tone period. The authors interpreted this suppression of response as an anxiety induced reduction in the strength of the hunger motivated lever pressing behaviour. Others have suggested that operant suppression results from the autonomic or skeletal responses to the conditioned stimulus which are incompatible with an operant task (Blackman, 1972). Hunt & Brady (1951) have termed the behavioural consequences of a conditioned stimulus (CS), a conditioned emotional response (CER) because overt responses indicative of changes in emotional state are induced by such a stimulus. The suppression of lever response provides a quantitative measure of the CER. Treatments which alleviate the CER without affecting the baseline response level before presentation of the CS are interpreted as anxiolytic. An anxiogenic effect would be reflected by a further suppression of lever response during application of the CS. To overcome the problem of detecting a reduction in a response which is already fully suppressed, the induction of a partial CER by varying the intensity of the unconditioned stimulus (i.e. shock; UCS), has been suggested (Millenson & Leslie, 1974).

A non operant version of this procedure uses the strength of the CER as an index of anxiety. The strength of the CER is determined by the recovery time from presentation of the UCS to resumption of the behaviour which was disrupted by the CS (Tenen, 1967).

1.2.d The drug discrimination paradigm: PTZ cue

A number of centrally acting drugs have been shown to induce interoceptive stimuli in animals which can be studied using operant conditioning procedures (see Lal, 1977). At subconvulsant doses, the drug PTZ is anxiogenic in man (Rodin, 1958). Accordingly, the interoceptive stimulus induced by subconvulsant doses of this drug in rats has been proposed as an animal analogue of anxiety (Shearman & Lal, 1979). As for the study of other drug induced stimuli, food deprived animals are trained to perform one operant task following drug administration and a different task following administration of the drug vehicle alone. Thereby, the subjects are conditioned to discriminate between the drug cue present and absent states. The operant schedules used to shape this discrimination require the animal to respond for food reinforcement on one of two levers in an operant chamber following either drug or vehicle administration. When such response differentiation is reliably established, the discriminative cue induced by PTZ is interpreted as an anxiogenic stimulus. Treatments which are anxiogenic, therefore, should produce a PTZ like response i.e. response on the PTZ appropriate lever, while treatments which are anxiolytic should antagonise the PTZ induced selection of the PTZ lever and instead result in selection of the vehicle appropriate lever. This paradigm has been established for rats (Shearman & Lal, 1979) and baboons (Ator et al., 1989) and is discussed in more detail in Chapters 5 and 6 where attempts to establish and evaluate this procedure as a model of anxiety in the pig are described.

1.3 The $GABA_A$/benzodiazepine receptor complex

1.3.a Benzodiazepine receptors in the CNS: pharmacological specificity and distribution

Pharmacological specificity

High affinity, saturable and stereospecific binding sites for benzodiazepines were demonstrated in the mammalian CNS by equilibrium binding techniques using tritium labelled 1,4-benzodiazepines (Squires & Braestrup, 1977; Möhler & Okada, 1977; Braestrup & Squires, 1977; Braestrup et al., 1977; Speth et al., 1978; Chang & Snyder, 1978). The finding of significant correlations between the ability of a series of benzodiazepines to displace [^{3}H]-diazepam binding from these sites and measures of their clinical and behavioural pharmacological potencies, indicated that the benzodiazepine binding sites mediate the pharmacological actions of the benzodiazepine compounds (Möhler & Okada, 1977; Speth et al., 1978; Lippa et al., 1978; Braestrup & Squires, 1978).

Distribution

Microdissection and autoradiographic techniques have demonstrated an uneven distribution of CNS benzodiazepine receptors. The highest concentrations in rat, mouse and human brain occur in the cerebral cortex and colliculi, with intermediate levels in the cerebellum, thalamus, striatum and hippocampus, and lowest levels in the pons, medulla oblongata and spinal cord (Squires & Braestrup, 1977; Möhler & Okada, 1977; Braestrup & Squires, 1977; Speth et al., 1978; Young & Kuhar, 1979, 1980; Biscoe et al., 1984). Using subcellular fractionation studies, the highest concentration of the binding sites were found in the synaptosomal fraction (Bosman et al., 1977).

In addition to the neuronal benzodiazepine receptor, another class of benzodiazepine receptor has been identified. Such receptors occur in a number of peripheral tissues eg. kidney and liver, and also in neural tissue on astroglial cells (Braestrup & Squires, 1977; Gallager et al., 1981). The two types of receptors can be distinguished by their preferential affinities for

different benzodiazepine ligands. The benzodiazepine clonazepam binds selectively to the neuronal site while PK 11195 is selective for the peripheral site. The peripheral site is poorly characterised and as yet its function is unknown although it appears to be localised in mitochondria and has become known as the mitochondrial benzodiazepine receptor (Anholt et al., 1986). In the present review, benzodiazepine receptor will refer to the neuronal benzodiazepine binding site.

A good correlation exists between the brain regions implicated in the modulation of anxiety and the distribution of the benzodiazepine receptors. High densities of benzodiazepine receptors occur in much of the limbic system, including parts of the amygdaloid complex, hippocampal formation, mammillary bodies and hypothalamus (Young & Kuhar, 1980). On the basis of neuroanatomical and clinical observations, Papez (1937) proposed a neural circuit of emotion which incorporated hippocampus, anterior thalamus, mammillary body, cingulate cortex and entorhinal cortex. Studies on the effects of brain lesions on emotional and conflict behaviour in animals have implicated the amygdala as an important site in the control of emotion. Thus animals with lesions of the amygdala fail to show normal conditioned avoidance learning, conditioned emotional responses, normal neophobia or learned aversion (see Rolls, 1975). Direct administration of benzodiazepines into the amygdala has been shown to produce an anticonflict effect in animals similar to that induced by peripheral administration of these drugs (Shibata et al., 1980; Petersen et al., 1985; Nagy et al., 1979; ScheelKrüger & Petersen, 1982; Thomas et al., 1985). The lateral amygdaloid nucleus was shown to contain the highest levels of amygdaloid benzodiazepine binding sites and also to mediate the anticonflict action of chlordiazepoxide in the rat (Thomas et al., 1985).

The above results, together with the finding that benzodiazepines depress both evoked and spontaneous firing rates of neurones in the septum, amygdala and hippocampus (Schallek & Kuehn, 1960; Chou & Wang, 1977) suggest that the benzodiazepines may exert their anti-anxiety effects through an action at the benzodiazepine receptor, which results in suppression of the limbic system.

1.3.b Drug and steroid interactions at the $GABA_A$/benzodiazepine receptor complex

GABA is the most prevalent inhibitory neurotransmitter in the mammalian CNS and is known to interact with at least two types of receptor, the so-called $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptors, defined by their susceptibility to the specific antagonist bicuculline, decrease neuronal activity by opening a Cl^- channel in the cell membrane, a channel which is an integral part of this receptor (see Olsen & Tobin, 1990). The first insight into the possible mechanism of action of the benzodiazepines came from electrophysiological studies which implicated a functional link between GABA and the benzodiazepines. These studies demonstrated a diazepam potentiation of presynaptic inhibition in the spinal cord (Schmidt et al., 1967) which could be blocked by pretreatment of the animal with GABA synthesis inhibitors or bicuculline (Polc et al., 1974). Subsequent electrophysiological studies confirmed a benzodiazepine induced enhancement of GABAergic inhibition (see Haefely, 1985). This enhancement was shown to be due to a benzodiazepine induced increase in the frequency of chloride channel opening in response to a GABA stimulus (Study & Barker, 1981).

A possible explanation for these electrophysiological findings was provided by biochemical data which indicated an association between GABA and benzodiazepine receptors. Skerritt & Johnston (1983) showed that diazepam was able to increase the low affinity binding of GABA to its receptor while earlier work had revealed that high concentrations of GABA were able to increase the affinity of the benzodiazepines for their binding sites (Tallman et al., 1978; Martin & Candy, 1978). Chloride and other anions which permeate the chloride channel enhanced the binding of [^{3}H]-benzodiazepines to the benzodiazepine receptor (see Olsen, 1982), supporting the association of the benzodiazepine receptor with the chloride channel coupled to the $GABA_A$ receptor. The hypothesis that this association with the $GABA_A$/chloride channel complex underlies the ability of the benzodiazepines to enhance the actions of GABA was further supported by the finding that specific benzodiazepine binding sites could be solubilised and co-purified with binding sites for the

$GABA_A$ receptor ligand muscimol (Gavish & Snyder, 1981; Sigel et al., 1982).

The discovery of ligands which bind to the benzodiazepine receptor but produce effects opposite or different to those of the 1,4-benzodiazepines led to the terminology of receptor agonists, antagonists and inverse agonists to account for the efficacy of these drugs at the receptor (Polc et al., 1982). Ligands which enhance GABA neurotransmission and produce the pharmacological profile of anticonvulsant, anxiolytic, muscle relaxant and sedative effects are termed agonists while those which produce an opposite profile are termed inverse agonists. Ligands which can antagonise the effects of agonists or inverse agonists but are without intrinsic activity themselves are termed antagonists. Other ligands exist which demonstrate restricted agonist or inverse agonist profiles and these are termed partial agonists and partial inverse agonists, respectively (Polc et al., 1982; Braestrup et al., 1982 and see Braestrup et al., 1984).

With the discovery of non-benzodiazepine ligands came the first evidence of benzodiazepine receptor heterogeneity. It was revealed that two classes of compounds, the triazolopyridazines and the ß-carbolines could displace [^{3}H]-diazepam and [^{3}H]-flunitrazepam binding with affinities and Hill coefficients that varied in different brain regions and these findings led to the classification of type 1 and type 2 receptors. The cerebellum was proposed to contain predominantly type 1 receptors, which display a high affinity for the ß-carbolines and triazolopyridazines while the hippocampus also contains type 2 receptors which display a lower affinity for these compounds (see Haefely et al., 1985).

The imidazobenzodiazepine Ro15-1788 (flumazenil) binds with high affinity to the benzodiazepine receptor (Möhler et al., 1981). The binding of Ro15-1788 is not affected by GABA (Möhler & Richards, 1981) nor is the binding of GABA affected by Ro15-1788 (Skerritt & Johnston, 1983). Electrophysiological studies revealed that this ligand can antagonise the modulatory effects of benzodiazepine agonists and inverse agonists on the GABA response without exerting any effect itself (Polc et al., 1982; Little, 1984

and see Haefely et al., 1985) and therefore appears to be a pure antagonist.

The binding of the ß-carboline ligands ßCCE, FG7142, ßCCM and DMCM is reduced in the presence of the GABA agonist muscimol (Braestrup et al., 1982). This contrasts the effect of muscimol on the binding affinities for the classical benzodiazepine ligands and is consistent with electrophysiological data which demonstrate that these ß-carboline ligands reduce the effect of GABA on chloride channel opening (Polc et al., 1982, Little, 1984; Jensen & Lambert, 1984) and can therefore be classified as benzodiazepine receptor inverse agonists. Attempts to demonstrate a change in the binding of GABA by these ligands have been unsuccessful (Skerritt et al., 1983).

The effect of the GABA agonist muscimol on benzodiazepine receptor binding can be expressed as the so-called GABA ratio. This is the ratio of the IC_{50} in the absence of muscimol to the IC_{50} in the presence of muscimol. A muscimol enhancement of benzodiazepine receptor affinity is reflected by a ratio greater than 1, a reduction in affinity by a ratio less than 1 while a ratio of approximately 1 indicates no effect of muscimol on receptor affinity (Braestrup et al., 1982). Table 1.1 shows the GABA ratio for the following benzodiazepine receptor compounds, ß-carboline ligands (nos. 1-8), the imidazobenzodiazepine Ro15-1788, the pyrazoloquinoline CGS8216, the triazolopyridazine CL218872 and the 1,4-benzodiazepines diazepam and chlordiazepoxide. From a consideration of these data and the electrophysiological findings described above, the ß-carboline ligands DMCM, ßCCE, ßCCM and FG7142 would be expected to show inverse agonist activity while the ß-carbolines ßCCP, ZK91296, ZK93426, the pyrazoloquinoline CGS8216 and Ro15-1788 would be expected to behave as antagonists and the triazolopyridazine CL218872 and the ß-carboline ZK93423 would be expected to have an agonist like profile.

The ß-carbolines DMCM and ßCCM are convulsant in mice and rats (Braestrup et al., 1982) while ßCCE and FG7142 only exhibit proconvulsant activity in these species (Nutt, 1983; Oakley & Jones, 1980; Little et al., 1984;

Corda et al., 1987). In man FG7142 is anxiogenic (Dorow et al., 1983). The imidazobenzodiazepine Ro15-1788 has been shown to antagonise the pharmacological effects of both agonists and inverse agonists (see Haefely et al., 1985). While the majority of clinical and behavioural data support the notion that this ligand is an antagonist at the benzodiazepine receptor, there are some reports of agonist and inverse agonist activity (see File & Pellow, 1986). The effect of the above ß-carbolines, Ro15-1788 and the other ligands displayed in Table 1.1, on animal analogues of anxiety is shown in Table 1.2. In general, there is a good agreement between the anxiolytic or anxiogenic effects of these drugs and their agonist or inverse agonist nature, as predicted by the GABA ratio. However, there are a number of compounds which may be classified as an anxiolytic or anxiogenic according to one test but appear to have no activity in another test. These discrepancies are particular to those compounds predicted to behave as receptor antagonists e.g. Ro15-1788.

Several models have been proposed to rationalise the diverse behavioural pharmacological data obtained from benzodiazepine ligands which act through a common receptor. One of these models proposes the existence of an endogenous ligand for the receptor. This ligand would occupy the receptor as a partial agonist and thereby impose some tone on the receptor system. Two diametrically opposite actions mediated through the same receptor could then be explained by the displacement of the natural ligand with a compound of higher or lower efficacy (Braestrup et al., 1984). Several compounds have been suggested as endogenous ligands for the benzodiazepine receptor including the purines, inosine and hypoxanthine, the ß-carboline harmane and a 105 amino acid peptide isolated from rat brain, called diazepam binding inhibitor (DBI; Guidotti et al., 1983 and see Haefely et al., 1985). In the Vogel conflict test DBI was shown to have a proconflict effect after intracerebroventricular injection which was sensitive to antagonism by systemic diazepam (Guidotti et al., 1983).

An alternative three state model has been put forward which assumes that the benzodiazepine receptor can exist in three energy states and that it can function as a coupling unit between GABA and chloride channels.

According to this model the binding of agonists or inverse agonists enhance or depress respectively the functional coupling by inducing a change in the receptor conformation while antagonists have no effect on receptor conformation but block access of both agonists and inverse agonists (Polc et al., 1982).

Table 1.1 The ratio of the IC_{50} for inhibition of benzodiazepine receptor binding by various ligands in the presence or absence of muscimol.

	Ligand	GABA ratio	Reference
1	DMCM	0.41	Braestrup et al.,1982
2	ßCCM	0.61	""
3	ßCCE	0.86	""
4	FG7142	0.87	""
5	ßCCP	1.11	""
6	ZK91296	1.23	Stephens et al.,1987
7	ZK93426	1.37	""
8	ZK93423	2.17	""
9	CGS8216	1.15	""
10	Ro15-1788	1.22	Braestrup et al.,1982
11	CL218872	1.98	""
12	Diazepam	2.3	""
13	Chlordiazepoxide	2.23	""

Biochemical and electrophysiological evidence supports the existence of several other sites on the $GABA_A$/benzodiazepine receptor complex which also act allosterically with each other and with the GABA and benzodiazepine sites. Additional sites implicated include those for the barbiturates, the convulsant drugs picrotoxin and t-butylbicyclophosphorothionate (TBPS) and for certain steroids; they will now be considered in turn.

Barbiturates have been shown to enhance postsynaptic responses to

GABA by increasing the lifetime of GABA activated chloride channels (Study & Barker, 1981), to enhance the binding of [^{3}H]-diazepam and [^{3}H]-GABA in a chloride dependent, picrotoxin sensitive manner and to allosterically inhibit picrotoxin and TBPS binding (see Olsen 1982; Ramanjaneyulu & Ticku, 1983, 1984).

The convulsant drugs picrotoxin and TBPS are thought to block the Cl^- ionophore, perhaps indirectly through binding at a closely located site (see Bormann, 1988). Binding of [^{35}S]-TBPS is inhibited by GABA agonists and barbiturates (Ramanjeyulu & Ticku, 1983, 1984) and enhanced by benzodiazepine agonists (Supavilai & Karobath, 1983). The ß-carbolines ßCCE, FG7142, ßCCM and DMCM reduce TBPS binding while the imidazobenzodiazepine Ro15-1788 has no marked effect on this parameter (Supavilai & Karobath, 1983). Some evidence suggests that the picrotoxin/TBPS site mediates the convulsant action of the drug PTZ. For example convulsant tetrazoles including PTZ displace [^{35}S]-TBPS binding competitively with a significant correlation observed between potencies to displace binding in vitro and convulsant potencies in vivo (Squires et al., 1984). It is known that PTZ antagonises GABA synaptic transmission (see Olsen, 1981) and further support for the hypothesis that this drug acts through the picrotoxin/TBPS site comes from dissociation studies with [^{35}S]-TBPS binding where PTZ displayed identical monophasic dissociation patterns to TBPS (Maksay & Ticku, 1985). Fluctuation analysis performed on cultures of mouse spinal neurons showed that PTZ decreased the lifetime of GABA activated ion channels but this effect could not fully account for the PTZ induced depression in the amplitude of the current response (Barker et al., 1983). The precise mechanism, therefore, by which PTZ may induce convulsions or anxiety through this receptor complex is largely unknown.

It has long been known that many steroids in the pregnane series have hypnotic and anaesthetic activity (Selye, 1942; Gyermek et al., 1967). Synthetic steroids such as alphaxolone and naturally occurring metabolites of progesterone and deoxycorticosterone have been shown to stimulate both [^{3}H]-benzodiazepine and [^{3}H]-GABA agonist binding and to displace [^{35}S]-TBPS

binding with characteristics similar to those displayed by the barbiturates and with such modulatory actions requiring a precise structure activity relationship (Harrison & Simmonds, 1984; Majewska et al., 1986, Harrison et al., 1987). These compounds also potentiated GABA inhibition in rat cultured hippocampal and spinal neurons and slices of rat hippocampus and cuneate nucleus (Harrison et al., 1987; Turner et al., 1989; Harrison & Simmonds, 1984) and prolonged GABA mediated inhibitory postsynaptic currents in cultures of rat hippocampus (Harrison et al., 1987). At micromolar concentrations, these steroids were shown to directly activate a Cl^- conductance in the absence of GABA (Harrison et al., 1987). A study of single channel recordings from cell lines expressing the cloned receptor complex subunits (see next section) has demonstrated that the progesterone metabolite 3α-hydroxy 5α-pregnan-20-one (3α-OH-DHP) and the deoxycorticosterone metabolite 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC) enhance GABAergic effect through an increase in the frequency of chloride channel opening (Puia et al., 1990). The important physiological implications of the above steroidal interactions with the $GABA_A$ receptor complex derives from the finding that some of these steroids (e.g. 3α-OH-DHP) are produced naturally during the ovarian cycle (Holzbauer, 1975; Ichikawa et al., 1974).

In contrast to the GABA enhancing effects displayed by the deoxycorticosterone and progesterone metabolites, the steroid pregnenolone sulphate appears to act as a GABA antagonist through the picrotoxin site. At μM concentrations, this steroid was shown to inhibit pentobarbital enhanced benzodiazepine binding and muscimol stimulated Cl^- uptake into synaptoneurosomes (Majewska & Schwartz, 1987). However, at nM concentrations, pregnenolone sulphate facilitated an increase in muscimol binding (Majewska et al., 1985). Evidence exists which suggests that pregnenolone may be synthesised in the brain (Corpechot et al., 1983) and this may be of physiological significance, in view of the above effects of the steroid on GABA function.

1.3.c Molecular biology of the $GABA_A$/benzodiazepine receptor complex

The $GABA_A$ receptor purified from mammalian brain by benzodiazepine affinity chromatography was found to have two major bands as determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis, corresponding to an α subunit of 53 kilodalton (KD) and a ß subunit of 56 KD. The molecular weight (MW) of the native receptor was estimated to be between 220 and 355 KD and the suggested stoichiometry was $\alpha_2\beta_2$. In the pure receptor the α subunit was photo-affinity labelled by flunitrazepam and the ß subunit was labelled by muscimol. Subsequent molecular cloning experiments led to the identification of 4 types of polypeptides, α, ß, γ and σ in the MW range of 50-60 KD with subtypes for each of these types, α_1-α_6, β_1-β_3 and γ_1-γ_2 (see Olsen & Tobin, 1990; Lüddens et al., 1990).

Expression of the cloned subunit sequences in Xenopus oocytes or mammalian cell lines produced functional GABA regulated chloride channels, but with variable pharmacological properties which were dependent on the subunit combination employed. Expression of mRNA for any individual subunit clone was able to produce GABA activated channels with pentobarbital and picrotoxin modulation but the γ_2 subtype was necessary for benzodiazepine modulation of the GABA response and for benzodiazepine binding. The pharmacological properties of benzodiazepine binding were shown to be dependent on the particular variant of the α subunit which is expressed. Co-expression of the α_1 subunit with the β_1 and γ_2 subunits resulted in binding properties characteristic of the type 1 receptor while expression of α_2 or α_3 with this ßγ combination yielded receptors with binding properties characteristic of the type 2 receptors (see Olsen & Tobin, 1990). Co-expression of the α_6 subunit with the β_2 and γ_2 subunits produced receptors which bound with high affinity to the imidazobenzodiazepines Ro15-4513 and Ro15-1788 but not the 1,4-benzodiazepines (Lüddens et al., 1990).

Each cloned subunit sequence contains four putative membrane spanning domains (m1-m4) of 22-23 α helical residues with a long hydrophilic NH_2 terminus containing potential asparagine glycosylation sites and a

cysteine pair which is believed to help hold the peptide in a conformation appropriate for ligand binding. The COOH terminus is located at the extracellular hydrophilic segment. Available evidence suggests that the receptor complex is composed of two to four different polypeptides and a total of 4-5 subunits, with the membrane spanning domains of the subunits forming the wall of the ion channel (see Olsen & Tobin, 1990).

1.4 Effects of benzodiazepine ligands on neurotransmitter systems implicated in the expression of anxiety.

The evidence reviewed in the previous section demonstrates that benzodiazepine receptor ligands act through the $GABA_A$ receptor complex to influence GABA function. Attempts to define a role for GABA in the modulation of anxiety have been hindered by the metabolic instability and poor blood brain barrier permeability of systemically administered GABA mimetics. However, several studies have now demonstrated an apparent anxiolytic action of such compounds following intracerebroventricular administration (e.g. Scheel-Krüger & Petersen, 1982; Thiebot et al., 1980; Vellucci et al., 1988). The serotonergic and noradrenergic transmitter systems have also been implicated in the expression of anxiety and evidence exists which suggests that the modulatory effects of the benzodiazepine ligands on anxiety may be mediated through these neurotransmitter systems.

5-Hydroxytryptamine (5HT)

Neurons containing 5HT are largely confined to the raphé nuclei of the brain stem and from these nuclei the major 5HT ascending projections to the forebrain originates (see Fuxe et al., 1970). A reduction of 5HT function either through lesions of 5HT neurons or by an inhibitor of tryptophan hydroxylase (eg. with parachlorophenylalanine, PCPA) has been shown to induce an anti-conflict action in animal models (Tye et al., 1977, 1979). Such an anti-conflict effect was also obtained on microinjection of GABA or benzodiazepine agonists into the dorsal raphé nucleus (Thiebot et al., 1980). The ability of these compounds to reduce 5HT function has been revealed by biochemical and

electrophysiological studies. Benzodiazepines have been shown to reduce the turnover and release of 5HT in the brain (Wise et al., 1972; Jenner et al., 1975; Saner & Pletscher, 1979) and to inhibit the firing of 5HT neurons (Laurent et al., 1983). Benzodiazepines also enhanced the inhibition of 5HT neuronal firing produced by iontophoretic application of GABA to the dorsal raphé (Gallager, 1978).

While the above evidence implicates a role for the 5HT system in the mediation of the anxiolytic action of the benzodiazepine ligands, other reports suggest that this system may also be involved in the anxiogenic actions of benzodiazepine inverse agonists. Injection of the ß-carboline ßCCM into the dorsal raphé nucleus produced a decrease in social interaction in rats and injection of Ro15-1788 into this region antagonised the decrease in social interaction induced by a systemic injection of ßCCM (see Stephens et al., 1986). However, while PCPA was shown to induce an anti-conflict action in mice subjected to the four plate test, this agent failed to antagonise the proconflict effect of the ß-carboline DMCM as did several 5HT receptor antagonists (Stephens et al., 1986). From these findings it was suggested that the 5HT system is not central to the mediation of the anxiogenic effects of benzodiazepine ligands. A similar conclusion could be drawn for the anxiolytic action of the benzodiazepines since an anxiolytic effect of 5HT receptor antagonism has not been consistently demonstrated. However, the 5HT receptor antagonists employed in such studies have known effects on other neurotransmitter systems and therefore the discrepancies which arise from these studies may reflect the lack of specificity of the 5HT ligands (see Chopin & Briley, 1987).

From the above it is evident that the 5HT system is involved in the anxiety modulating effects of the benzodiazepine ligands but the extent of this involvement remains unclear.

Noradrenaline (NA)

The source of the majority of the NA neurons in the brain is the locus coeruleus (see Fuxe et al., 1970). Electrical stimulation of this brain region was

shown to induce behavioural changes in monkeys similar to those evoked by threatening stimuli. These effects were antagonised by pretreatment with diazepam or the α2 adrenoceptor agonist clonidine (see Charney & Redmond, 1983). The α2 adrenoceptor antagonist yohimbine was shown to induce anxiety in man which was sensitive to alleviation by treatment with diazepam or clonidine (Charney et al., 1983). Some insight into the mechanism by which diazepam produced the above anxiolytic effects comes from electrophysiological studies which revealed a benzodiazepine induced reduction in the rate of firing of neurons in the locus coeruleus (Laurent et al., 1983). Biochemically benzodiazepines have been shown to decrease NA turnover but following chronic treatment with a benzodiazepine agonist this effect underwent tolerance and was therefore thought to be related to the sedative rather than the anxiolytic action of the benzodiazepines (Wise et al., 1972).

A role for the NA system in the mediation of benzodiazepine effects on anxiety is further suggested from the work of Gray (see Gray, 1983). The model of anxiety employed by this author is the septo-hippocampal theta rhythm. This model is based on the finding that lesions of the septum or hippocampus in the rat result in patterns of behaviour indicative of anxiety and stimulation of the septum (within the physiological range of theta frequencies) also induces such behaviour. Pharmacological blockade of NA transmission or lesions of the dorsal NA bundle abolish the minimum frequency threshold to drive the theta rhythm and this effect is also achieved with benzodiazepine tranquillisers.

However, evidence also exists which does not support a leading role for the NA system in the mediation of the anxiolytic or anxiogenic actions of benzodiazepine ligands. The α2 agonist clonidine did not induce an anticonflict response in mice in the four plate test and this drug only partially antagonised the proconflict effect of DMCM in the same paradigm (Stephens et al, 1986). Furthermore, depletion of central NA through lesions of the locus coeruleus or dorsal NA bundle did not induce an anticonflict action and such lesions did not influence the anticonflict action of a benzodiazepine (see Iversen, 1983; Koob et al., 1984). Thus, it would appear that not all aspects of anxiety modulated by the benzodiazepine ligands are dependent on the functional integrity of the

NA system.

1.5 The validity of animal tests as models of anxiety

On the basis of the pharmacological data presented in Table 1.2, an assessment of the reliability of a variety tests (described in section 1.2) in detecting compounds with anxiolytic or anxiogenic activity reveals the following: (1) Of those tests based on rodent exploratory behaviour, the elevated plus maze displays the greatest sensitivity in the detection of both anxiolytic and anxiogenic compounds. Pharmacological evaluations of this test have been largely confined to the rat. This is also the case for the social interaction test which is also sensitive to the detection of anxiogenic agents, but in naive animals the detection of anxiolytic activity is confounded by a greater sensitivity of this test to the sedative action of benzodiazepines (File et al., 1976). The holeboard test is also sensitive to the sedative action of benzodiazepines in the naive rat and the use of the test with this species has produced an anxiolytic classification of compounds such as Ro15-1788 and ZK93426, which is in disagreement with findings from other tests in the same species. In the mouse, this test is sensitive to the detection of anxiolytic benzodiazepines but the detection of anxiogenic activity of compounds such as FG7142 is confounded by a sensitivity to a non-specific drug induced change in locomotor behaviour (Lister, 1987a). As for the holeboard test, the light/dark choice exploratory test is sensitive to the detection of anxiolytic agents in the mouse but less so to anxiogenic agents. One report of a ßCCM induced change in test parameters indicative of anxiogenic activity is confounded by the concurrent finding that proconvulsant behaviour occurred at this test dose (Belzung et al., 1987).

(2) Of those tests based on punishment-induced conflict, the Vogel drink conflict test in the rat displays the greatest sensitivity in the detection of both anxiolytic and anxiogenic agents. The four plate test in mice is more sensitive to the detection of anxiolytic than anxiogenic activity. The Geller-Seifter test is sensitive to the detection of benzodiazepines with anxiolytic activity although in some cases these drugs also interfered with the control unpunished schedule

(Geller et al., 1962; Geller, 1964). A modified version of this procedure detected an anxiogenic action of ßCCM in mice, but the drug induced change in operant behaviour was not limited to the punished period (De Carvalho et al., 1983).

(3) The CER procedure is sensitive to the detection of benzodiazepine anxiolytics. Despite the suggestion that this test could be used to study anxiogenic agents by induction of a partial CER (Millenson & Leslie, 1974), such a study has not been reported for the benzodiazepine inverse agonists, possibly due to the inconvenience of rapid extinction.

(4) The PTZ drug discrimination paradigm is sensitive to detection of both anxiolytic and anxiogenic benzodiazepine ligands.

A major conclusion which can be drawn from the above assessment is that the various animal tests show different sensitivities to the detection of both anxiolytic and anxiogenic compounds. Three possible explanations may be put forward to explain this finding: that the different animal tests measure different aspects of anxiety, that the detection of a change in anxiety by some tests is confounded by a greater sensitivity of that test for other properties of the test drug, that the test is not measuring anxiety and any positive results obtained by this test reflect a detection of some other property of the drug.

Since most of the discrepancies between tests arise from the detection of anxiogenic activity, one implication of the first possibility is that some aspects of anxiety are largely not mediated through the $GABA_A$/benzodiazepine receptor complex, since anxiety in some test situations can be enhanced by benzodiazepine receptor inverse agonists but left unaffected in others.

From the description of the tests of anxiety in Section 1.2, it is clear that for the majority of these tests, the necessity exists to distinguish a drug effect on anxiety from a debilitating or secondary non-specific effect on locomotor behaviour. This necessity arises due to the dependence of the indices of anxiety measured on motor performance. While a distinction can be made between those drugs which affect the defined indices of anxiety only and those which

affect overall motor activity, the anxiolytic or anxiogenic nature of the latter remains inconclusive. In the social interaction test, benzodiazepine anxiolytic activity is not detected until the animals are made tolerant to the sedative effect of the drug (File et al., 1976; De Angelis & File, 1979). The PTZ discrimination paradigm is largely exempt from such problems since the index of anxiety measured is the direction rather than the frequency of the response.

Although a test may be shown reliably to detect compounds with anxiolytic or anxiogenic action, the pharmacological specificity of this test must be determined to ascertain its validity as an animal model of anxiety. If non-anxiolytic or non-anxiogenic compounds cause a change in test parameters interpretable as anxiolytic or anxiogenic, then the value of the test as a model of anxiety is equivocal. Of the tests described in this review, the four plate test is reported to generate many false positives (Aron et al., 1971).

In addition to pharmacological specificity, the validity of a particular model of anxiety is also dependent on the design of the test. It is evident from above that tests whose indices of anxiety correlate with motor performance are subject to many difficulties when interpreting anxiolytic or anxiogenic effects. Tests which are based on exploratory behaviour are particularly susceptible to such problems. The formidable task of distinguishing non-specific locomotor behaviour from true exploratory behaviour has been instrumental in the declined use of the classical model of exploration, the open field test. For some of the tests of exploration described here, attempts have been made to overcome such problems by monitoring task specific exploration e.g. time spent head-dipping in holes with novel objects (File & Wardill, 1975), time spent on the open arms of a plus maze (Pellow et al., 1985). In addition, behavioural evaluations have proved useful in defining those aspects of the animal's behaviour which specifically relate to exploration. According to Halliday (1968), exploration should lead to information storage and this should be reflected in the test measures as the animals are habituated to the apparatus. Such an evaluation of the holeboard test has revealed that the duration of head-dips shows a decrease on habituation unless novelty is reintroduced by placing objects in the holes (File & Wardill, 1975).

The indices of anxiety measured by the Geller-Seifter test, the four plate test and the CER also rely on motor performance and therefore these tests are subject to some of the difficulties encountered by tests of exploration. The specific type of reinforcement schedule employed in the Geller-Seifter and CER procedures may have important implications when interpreting the effect of drugs on these paradigms. It has been demonstrated that the CER is more severe when reinforcement schedules require high response rates rather than low response rates. The anxiolytic or anxiogenic nature, therefore, of drug treatments which exert opposite effects on control response rates and response suppression during the CS period, is equivocal (see Blackman, 1972). In addition, drug treatments are known to exert different quantitative and qualitative effects on different reinforcement schedules (see Dews & De Weese, 1977). This finding demonstrates the inadequacy of the use of the VI schedule in the conventional Geller-Seifter paradigm, as a control for non-specific side effects of drugs. Modifications of this procedure which largely overcome such ambiguities in interpretation of drug effects on control and conflict periods, include the use of multiple schedules, one of which induces a low response rate, comparable to that typically found during the conflict period (Tye et al., 1977, 1979).

Since the Geller-Seifter test, the four plate test and the Vogel conflict test employ electric shocks to induce conflict, anxiolytic or anxiogenic effects must be distinguished from effects on sensory threshold. Thiebot et al. (1980) using a modification of the Geller-Seifter procedure, have attempted to circumvent this problem by monitoring the effects of treatments on the conditioned conflict in the absence of electric shocks. Since the procedure then resembles the CER paradigm, caution must be taken in the interpretation of anxiolytic or anxiogenic effects of treatments which also influence the control response rate.

The Geller-Seifter test, the Vogel conflict test, the CER paradigm and the PTZ discrimination procedure all operate using food or water deprived animals. An interpretation, therefore, of results from these tests must take into account the possible interference with the motivational state of the animal. A

further complexity is added through the finding that benzodiazepine agonists and inverse agonists have opposite effects on food appetitive behaviour (Cooper, 1985a). To a large extent the drug discrimination procedure overcomes this problem since animals are trained to discriminate chemically induced anxiety from the non-anxious state under similar food deprived conditions.

A further criticism of the Vogel conflict test, the Geller Seifter test, the four plate test and the social interaction test is that they rely on different behavioural baselines to detect an anxiolytic and an anxiogenic effect.

Tests which rely on conditioned response suppression as an index of anxiety, for example the CER paradigm, generate problems in the interpretation of anxiolytic or anxiogenic effects of drug treatments which have known amnesic or promnesic actions. For instance, the benzodiazepine ligands diazepam and ß-CCM have been shown to exert opposite effects on memory and learning in rodents (Venault et al., 1986) and such properties of these drugs could account for a drug-induced attenuation or enhancement of conditioned response suppression, normally interpreted as an anxiolytic or anxiogenic action.

From the above it is clear that the PTZ discrimination paradigm is not subject to many of the limitations of the other tests. The validity of this test as a model of anxiety is dependent on the specific anxiogenic nature of the PTZ discriminative stimulus and this is discussed further in Chapters 5 and 6.

In conclusion, a pharmacological and behavioural evaluation of an animal test of anxiety should ideally satisfy the following criteria: a reliable and specific detection of anxiolytic or anxiogenic drug effects, an interpretation of behavioural parameter changes as anxiolytic or anxiogenic unconfounded by non-specific effects such as locomotor effects or effects on sensory or motivational thresholds. From the evidence discussed above it is clear that many of the available animal tests do not meet these criteria. Despite this finding, a combination of such tests has been useful in screening novel compounds for potential anxiolytic or anxiogenic activity. However, due to the

lack of specificity and other limitations of some of these tests they are inappropriate for studies on the neurobiology of anxiety or the interoceptive or exteroceptive stimuli which influence this state.

Table 1.2 Effects of GABA/benzodiazepine receptor ligands on animal tests of anxiety; + anxiolytic, - anxiogenic, O no effect, N.S. not significant.

Test	Animal	Drug	Dosage (mg/kg and route of administration)	Drug effect	Comment	Reference
Geller-Seifter	rat	Chlordiazepoxide	15 i.p.	+	Decrease in control response rate	Geller et al.,1962 Geller, 1964
Vogel	rat		8 i.p.	+		Vogel et al.,1971
Four plate	mouse		1, 2, 4 8, 16 32 i.p.	O + O	 reduced locomotion	Boissier et al.,1968
Holeboard	mouse		1.25, 2.5 5, 7.5 10 i.p.	O + O	16 hole apparatus	Nolan & Parkes,1973
	rat		5 i.p.		sedative	File & Lister,1982

Light/dark box	mouse	Chlordiazepoxide	0.5, 1 5, 10, 15, 20 50 i.p.	O +	sedative	Crawley & Goodwin, 1980
Social interaction	rat		2.5-7.5 i.p. (acute) 5 i.p.(chronic)	 +	sedative	File et al.,1976
Elevated + maze	mouse		5 10 i.p.	 +	Increase in no.& duration of open arm entries N.S.	Lister, 1987b
	rat		5 7.5 i.p.	+ +	N.S.reduction in total arm entries	Pellow et al.,1985
CER	rat		5-40 p.o.	+	increase in control response rate	Lauener, 1963
	rat		31.6 i.p.	+	sedative	Tenen, 1967

Test	Animal	Drug	Dosage	Effect	Comment	Reference
PTZ cue	rat	Chlordiazepoxide			% generalisation to PTZ cue	Vellucci et al.,1988
			0.16		60	
			0.32		55.5	
			0.625		56	
			1.25		21.4	
			2.5 i.p. +PTZ (20mg/kg,i.p.)		sedation	
	Rat		0.64-10 i.p.	+	ED_{50}= 1.8mg/kg	Shearman & Lal,1979
Geller Seifter	rat	Diazepam	15 i.p.	+	decrease in control response rate	Geller, 1964
	pig		0.25-2.5 s.c.	+		Dantzer & Roca,1974
	mouse		1 s.c.	+		De Carvalho et al., 1983

Vogel	rat	Diazepam	2, 4 i.p.	+		Vogel et al.,1971
	rat		0.5 i.v.	+		Corda et al.,1983
Four plate	mouse		0.5 1, 2 4 i.p.	O + +	 reduced locomotion	Boissier et al.,1968
Holeboard	mouse		0.75 1.25, 2.5 5 i.p.	O + O	16 hole apparatus	Nolan & Parkes, 1973
Light/dark box	mouse		0.5 2, 5 10 25 i.p.	 + O 	increased transitions N.S. sedative	Crawley, 1981
Social interaction	mouse		1 (acute) " (chronic) i.p.	 +	sedative	De Angelis & File, 1979
Elevated + maze	rat		1,2 i.p. (acute) "" (chronic) i.p.	+ +		Pellow et al., 1985

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Elevated + maze	rat	Diazepam	0.5 1, 2, 5 10 i.p.	O +	 sedative	Handley & Mithani, 1984
CER	monkey		4 s.c.	+		Holtzman & Villarreal,1969
	rat		17.8 i.p.	+	sedative	Tenen, 1967
PTZ cue	rat		 0.16 0.63 2.5 5 i.p. +PTZ (15 mg/kg)		% generalisation to PTZ cue 88 25 0 0	Stephens et al.,1984
	rat		1.25-10 i.p.	+	ED_{50}=3.4 mg/kg	Shearman & Lal, 1979
Elevated + maze	rat	CL218872	10, 20 i.p.	+		Pellow & File, 1986a

PTZ cue	rat	CL218872	ED_{50}=2.5 i.p.	+		Stephens et al., 1987
Vogel	rat	ZK93423	6 i.p.	+	MED=0.3 mg/kg	Stephens et al.,1987
Four plate	mouse		0.39, 1.56 3.13, 6.26 25 i.p.	O +	 decrease in unpunished crossings	Stephens & Kehr, 1985
PTZ cue	rat		 0.025 0.1 0.4 1.6 i.p. +PTZ 15mg/kg		% generalisation to PTZ cue 75 63 14 sedation ED_{50}=0.1	Stephens et al., 1984
Geller Seifter	rat	ZK91296	MED=12.5 i.p.	+		Petersen et al., 1984

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Vogel	rat	ZK91296	60 i.p.	O		Stephens et al., 1987
Four plate	mouse		0.78 3.13, 6.25, 25 i.p.	O +		Stephens & Kehr, 1985
Holeboard	rat		10, 20, 40 i.p.		decrease in rears sedative	Pellow & File, 1986b
Social interaction	rat		5 15 i.p.	+	 increase in s.i. N.S.	Pellow & File, 1986b
Elevated + maze	rat		5, 15 i.p.	O		Pellow & File, 1986b

PTZ cue	rat	ZK91296	 0.025 0.01 0.04 0.16 0.63 2.5 10 30 i.p. +PTZ 15mg/kg		% generalisation to PTZ cue 100 75 63 63 44 44 60 56	Stephens et al., 1984
Vogel	rat	ZK93426	3 i.p.	-		Stephens et al., 1987
Four plate	mouse		2.5, 10, 40 i.p.	O		Stephens & Kehr, 1985
Holeboard	rat		5 i.p.	+		File et al., 1986b
Social interaction	rat		2.5-10 i.p.	-		File et al., 1986b

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Elevated + maze	rat	ZK93426	5, 10 i.p.	O		Pellow & File 1986a
Geller Seifter	mouse	Ro15-1788	20 s.c.	O		De Carvalho et al., 1983
	rat		25 i.p.	O		Vellucci & Webster, 1982
Vogel	rat		2 i.v.	O		Corda et al., 1983
	rat		20, 30 i.p.	-		File & Pellow, 1985
Holeboard	rat		10 i.p. (acute) "" (chronic)	+	significant increase in all test parameters	File et al., 1986a
Light/dark box	mouse		1-50 i.p.	O		Crawley et al., 1984

Light/dark box	mouse	Ro15-1788	3 i.p.	O		Belzung et al., 1987
Social interaction	rat		4 10 20 i.p.	 - O	decrease in s.i. N.S.	File et al., 1982
Elevated + maze	rat		10, 20 i.p.	O		Pellow & File, 1986a
PTZ cue	baboon		0.1-10 p.o.	O		Ator & Griffiths, 1989
	rat		40 i.p.	O		Stephens et al, 1984
	rat		 2.5, 5, 10 20 40 i.p.		% generalisation to PTZ cue 0 16.7 50	Vellucci et al., 1988
Vogel	rat	CGS8216	1 i.v.	O		Corda et al., 1983

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Vogel	rat	CGS8216	2 i.p.	-		Petersen et al., 1983
Light/dark box	mouse		5-50 i.p.	O		Crawley et al., 1984
Social interaction	rat		10 i.p.	-		File & Pellow, 1984
Elevated + maze	rat		3, 10 i.p.	-		Pellow & File, 1986a
Holeboard	rat	ßCCP	4 i.v.	-		File et al., 1984
Social interaction	rat		2, 4 i.v.	-		File et al., 1984
Geller Seifter	rat	ßCCE	0.5-10 µg icv	O		Vellucci & Webster, 1982
Vogel	rat		0.2-0.4 i.v.	-		Corda et al., 1983
	rat		10 i.v.	-		Petersen et al., 1983

Holeboard	rat	ßCCE	1, 2 i.v.		decrease in head-dipping & locomotion	File & Lister, 1982
Social interaction	rat		1, 2 i.v.	-		File et al., 1982
PTZ cue	baboon		0.01, 0.032 0.1, 0.32 i.m.	O -		Ator et al., 1989
Vogel	rat	FG7142	10 i.p.	-		Stephens et al., 1987
	rat		2-5 i.v.	-		Corda et al., 1983
Four plate	mouse		6.25-100 i.p.		decrease in punished and unpunished crossings	Stephens & Kehr, 1985
Holeboard	mouse		5-40 i.p.		reduction in no. and duration of head dips and also locomotor activity	Lister, 1987a

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Light/dark box	mouse	FG7142	5-100 i.p.	O		Crawley et al., 1984
Social interaction	rat		5 i.p.	-		File & Pellow, 1984
Elevated + maze	mouse		5, 10 i.p.	-		Lister, 1987b
	rat		1, 5 i.p.	-		Pellow & File, 1986a

PTZ cue	rat	FG7142			% generalisation to PTZ cue	Vellucci et al., 1988
			2.5		8.4	
			3.75		27.3	
			5		77	
			7.5		22.2	
			10		33	
			20		0	
			40 i.p.		ataxic	
	rat				% generalisation to PTZ cue	Stephens et al., 1984
			2.5		25	
			5		75	
			7.5		45	
			10 i.p.		57	
					ED_{50}=5.0	
Geller Seifter	mouse	ßCCM	1 s.c.	-	decrease in control response	De Carvalho et al., 1983

Test	Animal	Drug	Dosage	Effect	Comment	Reference
Vogel	rat	ßCCM	0.1 0.3, 1, 3 i.v.	 -	N.S. reduction in punished licking	Nagatani et al., 1988
	rat		0.15 i.v.	-		Corda et al., 1983
Light/dark box	mouse		5 10, 20, 30, 40, 50 i.p.	O 	 significant decrease in transitions and locomotion	Crawley et al., 1984
	mouse		1 2.5 i.p.	 -	decrease in transitions N.S. proconvulsant behaviour	Belzung et al., 1987

PTZ cue	rat	ßCCM	 2 4 8 12 24 48 i.p.		% generalisation to PTZ cue 16.7 20 50 43 14.3 0	Vellucci et al., 1988
Vogel	rat	DMCM	0.5 i.p.	-		Stephens et al., 1987
	rat		0.5, 0.75 i.p.	-		Petersen et al., 1983
	rat		0.2 i.v.	-		Corda et al., 1983
Four plate	mouse		0.2, 0.8 3.1 i.p.	O	 decrease in punished crossings N.S.	Stephens & Kehr, 1985

Test	Animal	Drug	Dosage	Effect	Comment	Reference
PTZ cue	rat	DMCM	ED_{50}=0.3 i.p.	-		Stephens et al., 1987
Vogel	rat	PTZ	5 10, 20, 40 i.p.	 -	N.S. reduction in punished licking	Nagatani et al., 1988
	rat		15 25 s.c.	-	significant reduction in punished and unpunished licking	Petersen et al., 1983
	rat		15 i.p.	-		Corda et al., 1983

Holeboard	rat	PTZ	10 20 i.p.		N.S. decrease in no. and duration of head dips and increased locomotion significant decrease in no. of head dips and locomotion	Pellow et al., 1985
Social interaction	rat		5 10 20 i.p.	O	 decrease N.S. significant decrease in locomotion	File & Lister, 1984
Elevated + maze	rat		10, 20 i.p.		decrease in open and closed arm entries	Pellow et al., 1985

1.6 Conclusions

The ability of benzodiazepine receptor ligands to modulate clinical and behavioural manifestations of anxiety, together with the distribution of specific benzodiazepine receptors in those brain areas implicated in the regulation of this emotional state provide strong evidence that anxiety is mediated through the $GABA_A$/benzodiazepine receptor complex. Advances in the elucidation of the structure and function of the $GABA_A$ receptor complex together with the development of drugs which act through this receptor to exert bi-directional effects on anxiety has greatly facilitated our understanding of the neural mechanisms underlying the generation and modulation of anxiety. Crucial to this understanding is the development of animal analogues of anxiety. However, due to inappropriate design and poor pharmacological specificity, results obtained from some of the available tests require further validation prior to assigning an anxiolytic or anxiogenic role to a particular stimulus. While such tests have proved useful when combined with a battery of other tests in the determination of pharmacological correlates of drug effects in vitro, their application to the study of anxiety is limited.

The aim of the present project was to study hormonal and environmental influences on anxiety through the evaluation and development of two animal models of anxiety. The light/dark choice test of exploration was employed to study the effect of ovarian steroid hormones on GABA function and anxiety in the mouse. This test was chosen because it is sensitive to benzodiazepine anxiolytics in naive mice (Crawley, 1981) and also to the detection of changes in exploratory behaviour during the mouse oestrous cycle (Gray, 1978). In other experiments undertaken to study environmental influences on anxiety in the pig, the PTZ discrimination paradigm was employed since pigs provide ideal subjects for operant conditioning procedures (Baldwin & Stephens, 1973) and, as discussed in the previous section, the PTZ conditioning procedure has many advantages over other tests of anxiety. Attempts were made to evaluate these tests both behaviourally and pharmacologically.

Chapter 2

Materials and methods

2.1 Materials

- **2.1.a** Chemicals and reagents
- **2.1.b** Radiochemicals
- **2.1.c** Buffer solutions
- **2.1.d** Drugs

2.2 Treatments and behavioural tests applied to mice

- **2.2.a** Animals
- **2.2.b** Apparatus
- **2.2.c** General test procedure
- **2.2.d** Data analysis

2.3 Treatments and behavioural tests applied to pigs

- **2.3.a** Animals
- **2.3.b** Apparatus
- **2.3.c** Jugular catheterisation
- **2.3.d** Pharmacological conditioning procedures
- **2.3.e** Test procedures
- **2.3.f** Procedures used to examine the effect of a conditioned emotional state on the discriminatory response of pigs
- **2.3.g** Data analysis

2.4 Methods used in the determination of whole brain concentrations of diazepam

- **2.4.a** Preparation and extraction of mouse brain
- **2.4.b** Radio-immunoassay
- **2.4.c** Preparation of _Staphylococcus aureus_ cells
- **2.4.d** Protein assay : Dye binding method
- **2.4.e** Liquid scintillation counting
- **2.4.f** Data analysis

2.1 Materials

2.1.a Chemicals and reagents

Albumin, bovine serum, fraction V.............................. Sigma Chemical Co. Ltd.
Coomassie Brilliant Blue G..Sigma Chemical Co.Ltd.
Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid).........................
B.D.H. Chemicals Ltd.
Merthiolate (Thiomerosal,sodium ethylmercurithiosalicylate)...........................
Sigma Chemical Co.Ltd.
Propylene glycol (Propane-1,2-diol)..Fisons
Scintillation fluid:Optiphase X...L.K.B.
Staphylococcus aureus cells..gift: M.Ginsburg
(I.C.R.F.Laboratories)
Tris-(hydroxymethyl)-methylamine....................................B.D.H.Chemicals Ltd.

Acids and other chemicals routinely used in the laboratory were obtained from B.D.H. Chemicals Ltd.

2.1.b Radiochemicals

Flunitrazepam[N-methyl^{3}H],(78.9Ci/mmol)..............Amersham International Plc
n-Hexadecane [^{3}H],(2.07 uCi/g)............................... Amersham International Plc

2.1.c Buffer solutions

Phosphate buffered saline (PBS): pH 7.4 at 20°C.
di-Sodium hydrogen orthophosphate ($Na_2 HPO_4$): 40.5 mM; sodium chloride (NaCL):100 mM. Adjust to pH 7.4 with 0.2 M sodium dihydrogen orthophosphate, ($NaH_2 PO_4.2H_2O$): final concentration 9.5 mM.

PBS containing bovine serum albumin (0.1% w/v)and merthiolate (0.01% w/v) (PBS/BSA/M).

Hepes-Tris: pH 7.4 at 20°C

N-2 hydroxyethylpiperazine-N'-ethanesulphonic acid (0.5 M) adjusted to pH 7.4 with saturated Tris-(hydroxymethyl)-methylamine: final concentration 0.25 M.

Hepes-Tris containing bovine serum albumin (0.25% w/v) and merthiolate (0.025% w/v) (Hepes/Tris/BSA/M).

Standard phosphate buffers: for calibration of pH meter

(1) pH 6.84 at 38°C, a solution of potassium dihydrogen orthophosphate (KH_2PO_4) 3.4 g/L and di-sodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) 4.408 g/L

(2) pH 7.38 at 38°C, a solution of KH_2PO_4, 1.816 g/L and $Na_2HPO_4.2H_2O$, 9.504 g/L.

2.1.d Drugs

Azaperone....................Janssen Pharmaceutica
Benzylpenicillin....................Glaxo-Vet
d-Amphetamine sulphate....................Sigma Chemical Co.Ltd.
Diazepam....................gift: Roche Products Ltd.
Ethosuximide....................gift: Park-Davis & Co.
FG-7142....................gift: Schering AG
Heparin....................Glaxo-Vet
Metomidate hydrochloride....................Janssen Pharmaceutica
Pentylenetetrazole....................Sigma Chemical Co. Ltd.
Ro15-1788....................gift: Roche Products Ltd.

d-Amphetamine sulphate was dissolved in PBS at 0.2 mg/ml immediately before use.

Diazepam was dissolved initially in a propylene glycol/ethanol mixture (2:1 v/v) which was then diluted to 4% with PBS before use.

Pentylenetetrazole (PTZ) was dissolved in 150 mM NaCl immediately before use.

For oral administration, diazepam, ethosuximide, FG-7142 and Ro15-1788 were made up in a food paste which consisted of 16-20 gms of pig pellets and 16-20 ml of saccharin (0.1% w/v) dissolved in tap water.

2.2 Treatments and behavioural tests applied to mice

2.2.a Animals

Adult male and female C57BL/6J mice (16-30gm) bred in the Joint Animal House at University College London were used. They were kept in groups of 3 or 4 in standard plastic cages (280x158x127 mm) with food (RM 3 cubed pellets, Special Diet Services) and water ad lib. The animals were housed in the same room that was used for behavioural observations and allowed to adapt to this room for at least seven days before the start of an experiment. This room was maintained at 20-22°C on a 12 hour light/dark cycle, with the lights on between 06:00 and 18:00 hr or 09:00 and 21:00 hr and providing a maximum background illumination of 280 Lux. For one experiment in which behavioural tests were conducted during reversed daylight the lights were on between 23:00 and 11:00 hrs with red light (2x60 W) providing the background illumination necessary for testing.

Cages containing male or female mice were alternated in the cage racks to encourage regular oestrous cycles. The females were not used for experiments until they had shown at least two regular oestrous cycles, each of 4-5 days duration. Stages of the oestrous cycle were monitored by taking daily vaginal smears (Allen, 1922). A platinum loop was inserted 2mm on the vaginal wall and the epithelial cells obtained were stained on glass slides with toluidine blue (1% aqueous in PBS). The different cycle stages were distinguished according to the cell content of each smear as described by Allen (1922). Typical smears from each oestrous cycle stage contained the following cell types:

Pro-oestrus; mainly epithelial cells, few cornified, overall very few cells.

Oestrus; mainly cornified cells, some epithelial.

Metoestrus I; all cells cornified and clumped.

Metoestrus II; cornified cells, epithelial cells and leucocytes.

Dioestrus; epithelial cells, leucocytes and mucus.

Late Dioestrus; epithelial cells, few cornified cells,few leucocytes and some mucus.

Vaginal smears were taken daily from 09:00 to 11:00 hrs. On the day of the experiment a final smear was taken immediately after the behavioural test or before the animals were treated prior to removal of brains for the neurochemical study described in Section 2.4, to corroborate the cycle stage determined from the morning smear.

In two experiments, the handling female mice experienced from the taking of vaginal smears was mimicked in males over a period of 14 days by daily removing them from their home cages for a period of up to 10 seconds and then returning them.

Diazepam or the propylene glycol/ethanol/PBS vehicle was injected intraperitoneally at 10 ml/kg 60 minutes before behavioural testing or the removal of brains for the measurement of diazepam concentrations (Section 2.4). d-Amphetamine or its saline vehicle was injected intraperitoneally at 5 ml/kg 40 minutes before the behavioural test. The various treatments to be given (untreated, sham injection, propylene glycol/ethanol/PBS injection, diazepam injection, PBS injection or injection of d-amphetamine) were assigned at random to the experimental animals to be used in the behavioural test on each day. No less than 6 animals were used for each treatment group.

2.2.b Light/dark box test apparatus

The light/dark box exploratory apparatus is shown in Fig 2.1. The box was constructed from black perspex and consisted of two chambers connected by a tunnel (101x30x50 mm). The light chamber (295x210x210 mm) was open topped and evenly illuminated by a fluorescent lamp (2x15 W) situated 125 mm above the top of the box. The other chamber (148x210x210 mm) was darkened by a lid. Both chambers and tunnel had a clean sheet of matt black paper as a floor, which was replaced after each test. Photocells were mounted 3 mm above the floor of the box, immediately opposite a small infra-red light source

and set to detect interruptions of the light beam. There were two such photocells in the light chamber, four in the tunnel and one in the dark chamber and they were connected to a recording system monitoring both the overall locomotor activity of the mouse in each chamber and the number of transitions between the two chambers. In addition, this system was also set to monitor the amount of time the animal spent in each chamber.

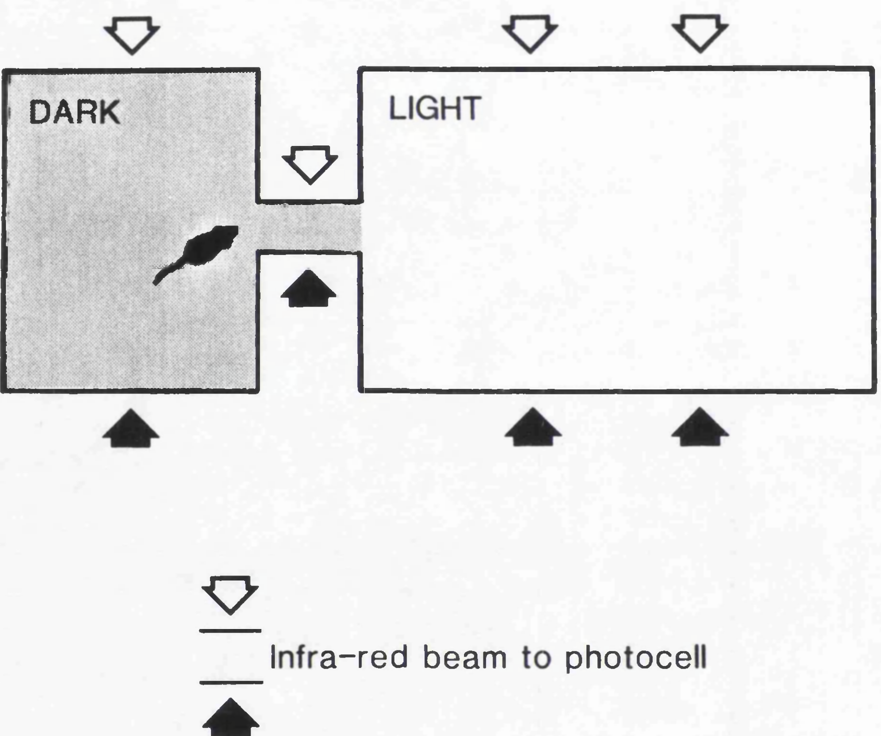


Fig. 2.1 The light/dark choice exploratory apparatus

2.2.c General test procedure

Subjects which were naive to the light/dark box apparatus were tested individually for a period of 15 minutes. Preliminary experiments had shown that this test duration produced the optimal difference in the number of light/dark tunnel transitions between mice treated with diazepam and those treated with the drug vehicle alone. Male and female mice were never tested in the apparatus on the same day and an interval of at least one day was allowed between male and female test days. On male test days, the first animal tested was always untreated and the data obtained from this test were not used in the final data analysis. Each test was initiated by placing the mouse in the dark chamber. For each experiment the test parameters measured were, the number of light/dark transitions, the time spent in the light chamber, the latency to first emerge from the dark chamber, the overall activity in each chamber and the number of rearings made in the light chamber. This last parameter was not monitored for tests conducted during reversed daylight as the light chamber was screened from the experimenter. Locomotor activity in light and dark chambers was normalised for each animal by expressing this parameter as a proportion of the time spent in the respective chambers. Tests were carried out between 12:00 and 19:00 hr.

Modifications of the test procedure were employed in an evaluation of this test and are described in Chapter 4.

2.2.d Data analysis

Statistical significance of differences in light/dark box test parameters between different treatment groups was determined using the Mann-Whitney U test (two tailed). A Kruskal-Wallis one way analysis of variance was used to test for any significant change in behavioural observations in female mice during the oestrous cycle.

2.3 Treatments and behavioural tests applied to pigs

2.3.a Animals

Male pigs (15-20 kg, initial weight) of the Large White breed, eight weeks old at the start of the study were used. They were bought from a commercial pig breeder and allowed to adapt to the change of housing conditions for one week before experimentation. They were housed individually in straw floored pens in a barn which was maintained at 18-20°C under normal daylight conditions and situated adjacent to the laboratory and conditioning room. The animals were fed a restricted diet of pellets ('spotlight' stage 1 pellets; Charnwood Milling Co. Ltd.) at 1.5-2% of body weight. At the end of each experimental day the animals received the amount of this food which was not consumed during conditioning experiments. Water was freely available in the home pens.

2.3.b Apparatus

The apparatus consisted of a modified Skinner box (136.5x76x62 cm; see Fig 2.2) constructed from sheet metal. A removable panel (69.9x56 cm) was fitted to each end of the box. The back panel functioned as the door of the box. To the front panel were attached two manipulanda (12x17 cm), a food bowl and a food dispenser stoppered at the bottom by a trap which was controlled by a solenoid. Each manipulandum had a lever switch (7.5x5.1 or 10x7 cm) for the pig to press. The system was interfaced to a computer programmed in Quick-Basic (West, D.C., Ephaptek) to record each lever press and with the facility to allow the operator to vary the size of the reward and the ratio of lever press to reward. A wire grid (25 mm mesh made from 5 mm gauge wire) was placed on the floor of the box which had a tilt in the centre leading to an opening for easy drainage while cleaning. The grid was at a distance of 27 cm from the ground. A wooden ramp positioned in the door allowed the animals to enter and leave the box. The box was housed in an insulated room (2.3x2.7x2.25 m), maintained at 20-22°C, illuminated by a 58 Watt fluorescent tube and with constant background noise provided by three ventilation fans.

(a)

(b)

Fig.2.2 Modified Skinner box: (a) apparatus and (b) pig in the box eating food reinforcement.

2.3.c Jugular catheterisation

The pigs were fitted with a polyvinyl catheter (external diameter 1.85 mm and internal diameter 1.5 mm; Portex Ltd.) in the external jugular vein via a ventral neck incision, under azaperone (2 mg/kg, i.m.)/metomidate(10 mg/kg, i.p.)/N_2O anaesthesia. The free end of the catheter was led subcutaneously to an opening behind the ear where it was connected with a catheter mount (Portex Ltd.) to a three way tap (Viggio Ltd.). The external tubing and three way tap were fixed in position on the animal's neck by means of elastoplast bandage such that the three way tap was situated at the centre of the neck behind the ears. Catheters were filled with a mixture of heparinised saline (200 units/ml) and benzylpenicillin (0.1 M unit/ml) which was replenished at least twice daily.

2.3.d Pharmacological conditioning procedures

Pigs were conditioned to discriminate the stimulus induced by the drug PTZ from saline. The procedures used to achieve this discrimination are described below.

1 Initial training

Following a short period of habituation (approx. 5 mins) to the Skinner box during which time food pellets were available in the food bowl, pigs were trained to press at both levers on a continuous reinforcement schedule where each lever press resulted in the delivery of 2-3 gms of pig pellet. A covering of food paste (pellets dissolved in tap water) enabled the animals rapidly to associate lever pressing with food reinforcement.

2A Conditioning procedure to produce selection of one lever only following either saline or PTZ treatment

When animals had been trained according to method 1 above, the ratio of lever presses to food reward was gradually increased for each lever from 1 to a fixed ratio of 40 (FR 40). This was generally achieved by increasing the ratio for both levers by 5-10 presses in individual sessions which usually lasted

30 mins. The food reward for a completed ratio on either lever was 3-4 gms of pellets. When the pigs were reliably responding on both levers at a FR 40, training was stopped to permit the surgical implantation of intravenous catheters (Section 2.3.c). After recovery from surgery (usually 2 days) the animals received several 10 minute training sessions (FR 40) in which only one lever was reinforced and the choice of this lever was alternated between sessions. This procedure was adopted to avoid the development of a lever preference.

The pigs were then trained to discriminate the effect of PTZ from saline. The training dose of PTZ was initially set at 5 mg/kg for each pig. Preliminary experiments had shown that pigs could recognise the PTZ stimulus when the drug was administered intravenously within a dose range of 5-10 mg/kg. The PTZ dose was increased from 5 mg/kg if the animals failed to show signs of discrimination after 5-6 training sessions. If at the increased dose, any drug induced behaviour indicative of a proconvulsant state became apparent such as tremor of the body or twitch of the head, the dose was subsequently decreased.

Pigs were trained to select one lever at a FR 40 following an intravenous injection of PTZ (1.5 ml followed by a 2 ml flush of heparinised saline) and to select the other lever (FR 40) following an intravenous injection of the same volume of the saline vehicle. For each pig the position of the drug and saline designated lever was kept constant for the course of the experiment. The right hand lever (lever B) was the designated drug lever for 2 pigs while the left hand lever (lever A) was the designated drug lever for the remaining three pigs.

Training sessions were conducted immediately following injection of drug or saline in the home pen. The order in which the animals were trained was randomised daily. PTZ and saline treatments were administered according to the following schedule, S P S S P P S S P S, where P denotes a PTZ training session and S a saline session. Both saline and PTZ training sessions lasted 10 minutes. All training sessions were initiated by placing 3-4 gms of food pellets

in the bowl before the animals had started to respond. This was done to orient the animals between the two levers at the start of each session.

The criterion of successful discrimination was 9/10 consecutive sessions for each treatment with no more than 4 presses on the non-rewarded lever before the first reinforcement.

2B Conditioning procedure to produce selection of both levers alternately following saline treatment and selection of one lever only following PTZ treatment

When pigs had been trained according to method 1 described above, the ratio of lever presses to food reward was gradually increased from 1 to a fixed ratio of 20 (FR 20). This was achieved by increasing the ratio of lever presses to reward, by 2-4 presses in individual sessions, usually of 30 min duration. During this training the levers were set to reward in an alternate fashion (alternation training) such that at the end of this phase the animals had learned that when reinforcement had been obtained by pressing one lever at a FR 20, the next food reward could only be obtained by pressing the alternate lever at a FR 20. Food reinforcement for a correct selection consisted of 2-3 gms of food pellets. After the animals had undergone and recovered from, the surgical implantation of intravenous catheters (Section 2.3.c), they received a further period of alternation training until they had satisfied a criterion of 9/10 consecutive 10 minute sessions in which there were no incorrect selections. An incorrect selection was defined as 5 or more presses on the non-rewarded lever.

The pigs were then trained to discriminate the effect of PTZ from saline. The training dose of PTZ was initially set at 10 mg/kg for each pig and subsequently reduced during the experiment such that on administration of drug to any animal there was no physical response such as tremor, which would suggest a proconvulsant state.

Pigs were trained to select one lever at a FR 20 following an intravenous injection of PTZ (1.5 ml followed by a 2 ml flush of heparinised saline) and to

select both levers alternately following an intravenous injection of the same volume of the saline vehicle. For each pig the position of the designated drug lever was kept constant for the course of the experiment.

Training sessions were conducted immediately following injection of PTZ or saline in the home pen. The order in which the animals were trained was randomised daily. Initially, several saline training sessions were included between drug training sessions until a saline session occurred, following a PTZ session, in which there were no incorrect selections. Then PTZ and saline treatments were administered according to the schedule, S P S S P P S S P S, where S denotes a saline training session and P a PTZ training session.

The saline training session time was fixed at 10 minutes for the entire experiment. The duration time of PTZ training sessions was initially set at 10 minutes but was subsequently reduced for each animal, following a tendency to switch to alternation of lever selection after a period of drug lever selection. This protocol was adopted to avoid training on the PTZ lever in the event of the PTZ stimulus being absent. All training sessions were initiated by placing 2-3 gms of food pellets in the food bowl.

The criterion of successful discrimination was 4/5 consecutive saline sessions with no more than one incorrect selection in each consecutive 10 selections and 4/5 consecutive PTZ sessions with no incorrect selections.

2.3.e Test procedures

When animals had been trained to discriminate PTZ from saline by the procedures described above, they were tested with other drugs to examine the pharmacological specificity of the PTZ stimulus by determining the ability of these drugs to generalise to or antagonise the PTZ stimulus.

Generalisation and antagonism tests using pigs conditioned by procedure 2A (Section 2.3.d).

As for training sessions, each test session was initiated by placing 3-4

gms of food pellets in the food bowl. Test sessions were terminated without reinforcement when forty presses had been completed on either lever. This protocol was adopted to avoid detraining the animals.

Generalisation tests:

FG7142 (5-40 mg/kg, p.o.) was administered in the pen 45 mins prior to a sham injection and the animals were tested immediately following this treatment.

Antagonism tests :

Diazepam (0.125-0.5 mg/kg, p.o.) was administered 30 minutes before an intravenous injection of the training dose of PTZ.

Ro15-1788 (5 mg/kg, p.o.) was administered 60 mins prior to an intravenous injection of the training dose of PTZ. The animals were treated with diazepam (0.5 mg/kg, p.o.) or the food paste alone 30 mins after administration of Ro15-1788. Tests were conducted immediately following the PTZ injection.

To avoid any possible interference from an association of a previous drug cue with food paste administration, control sessions were run in which the animals received the food paste alone and were tested after the same time periods as for drug administration. PTZ and saline training sessions were given between test sessions according to the training schedule. If animals failed to discriminate PTZ from saline in these sessions, the previous test result was considered invalid and the animals were retrained to satisfy a criterion of 4/5 consecutive sessions for each treatment with no more than 4 presses on the non-rewarded lever before the first reinforcement.

Discrimination, generalisation and antagonism tests using pigs conditioned by procedure 2B (Section 2.3.d)

Discrimination tests :

When the pigs satisfied the criterion of successful discrimination they were presented with the training dose of PTZ or saline during a saline session. Following saline administration in the home pen the animals were subjected to a 5 minute session in which only alternate selections were reinforced. Then the training dose of PTZ or the same volume of saline (2 ml) was infused by a remote-controlled pump (50 ml syringe, setting 10 (ml/min); Sage Instruments) at a rate of 0.22 ml/sec through a manometer line (100 cm,1.92 ml) which was louer locked onto the three-way tap connection of the catheter. The manometer line was filled with the PTZ or saline at the start of the session. The volume of the catheter, three-way tap and connection was 1.12 ml and therefore at this rate of infusion the PTZ or saline would begin to enter the vein after 5.04 secs. The total infusion time was 15 secs. The animals were habituated to wearing the manometer line for several saline training sessions before the test session. On infusion of PTZ or saline, both levers were set to reward and the test was terminated 5 mins after the start of infusion.

Generalisation and antagonism tests :

Each test session was initiated by placing 2-3 gms of food pellets in the food bowl. During test sessions both levers were set to reward. Training sessions were conducted between test sessions to ensure maintenance of the discriminatory response. If animals failed to discriminate PTZ from saline in these sessions then the previous test result was discarded and the animals were retrained to satisfy a criterion of 4/5 consecutive sessions within the discrimination criterion described in Section 2.3.d.

Generalisation test :

FG7142 (40 mg/kg, p.o.) or the food paste alone was administered in the pen and five test sessions of approximately 5 min duration were conducted following an intravenous saline injection, at 15 min intervals.

Antagonism tests :

Diazepam (0.5 mg/kg, p.o.) or the food paste alone was administered in the pen and the animals were tested after an intravenous saline injection 25 minutes later. Following a response period of 5 mins, the training dose of PTZ was administered as described above for discrimination tests.

Ethosuximide (30 mg/kg, p.o.) was administered in three doses of 10 mg/kg, each treatment separated by 60 mins. The animals received an intravenous saline injection and were tested 60 mins after the last treatment of drug or food paste alone . The training dose of PTZ was presented, as described above, after a response period of 5 mins.

Antagonism tests were usually terminated 5 mins after infusion of PTZ.

2.3.f Procedures used to examine the effect of a conditioned emotional state on the discriminatory response of pigs

Animals which were trained to discriminate PTZ from saline by method 2B (Section 2.3.d) were subjected to a modification of the procedure known to induce a conditioned emotional response in the pig (Baldwin and Stephens, 1973). A tone (1600 Hz) of 180 secs duration was conditioned (conditioned stimulus; CS) by pairing it with an electric shock (unconditioned stimulus; UCS).

Apparatus :

The tone apparatus consisted of a miniature solid state buzzer (RS Components Ltd.). The shock was delivered from an induction coil operated by batteries which was constructed from a modified commercial pig goad (Kawe Ltd.) which had an open circuit of 100 pulses per second. The buzzer and pig goad were activated from a control box constructed (Electronics Workshop, University College London) with a timing device which had settings for tone and shock duration and which ensured that termination of tone presentation resulted in the delivery of an electric shock for its preset duration. The control

box was interfaced to the computer and a modification of the experimental program allowed the operator to preset both the time of onset and duration of tone presentation.

Conditioning procedure :

At the end of daily training sessions the animals had been habituated for a period of at least two weeks, to receiving the remainder of their daily diet either in a temporary pen constructed in the conditioning room or in the Skinner box with the manipulanda removed. The animals were also accustomed to having pregelled silver/silver chloride electrodes (S & W Medico Teknik A/S) attached to their backs for the duration of these feeding times. The tone stimulus was conditioned in this non-operant situation to avoid undesired associations such as response on a particular lever with the delivery of shock. Following 1 min of undisturbed eating the tone was sounded for 180 secs and terminated by an electric shock of 150 msec duration, administered through the pregelled electrodes attached to the pig's back.

Test procedure :

The effect of the tone stimulus on the discriminatory response of animals pretreated with saline was examined. The buzzer was situated on the wall of the conditioning room above the front panel of the Skinner box, equidistant from both levers. The tone was presented for a duration of 180 seconds, 300 seconds following the start of a 10 min session. Prior to aversive conditioning, any tone induced disruption of the alternation response resulted in further saline/tone sessions until the tone stimulus had no effect on the saline response. Following presentation of the conditioned tone stimulus, both levers were set to reward for the remaining 300 secs of the test period. The saline/conditioned tone (S/CT) test sessions were incorporated into the training schedule as follows:

DAY 1	DAY 2	DAY 3	DAY 4
S	S	S/CT	P
P	S/CT	P	S/CT
S	P	S	S
S/CT		S	

Following conditioning of the tone stimulus the pigs received one S/CT session on each consecutive day, for the course of study of the discriminatory response to the state induced by this stimulus. The effect of diazepam on this response was examined by presentation of the conditioned tone stimulus as described above following a diazepam (0.5 mg/kg, p.o.; 30 mins) pretreatment.

2.3.g Data analysis

Data recorded from training and test experiments using pigs trained by procedure 2B (Section 2.3.d) were analysed by a program written in Quick-Basic. This program was designed to extract the information necessary for subsequent statistical analysis, in a spreadsheet (Excel, Microsoft Corporation) format (comma separating value (.csv) files).

Mean frequencies of response on both levers from saline and PTZ training sessions were compared using a two-sample t-test (two-tailed).

Statistical significance of differences in lever selections between different treatments applied to each pig was determined using the Fisher exact probability test (two-tailed). When stimuli were applied during a saline session, lever selections were analysed for the duration of the stimulus if there was a known period of effect or from the time of application until the end of the test if the applied stimulus did not have a fixed or determinable period of effect. In each case, the last lever selected before application of the stimulus was included in the analysis to give a more accurate determination of any stimulus induced change in the animal's behaviour. Lever selections were considered in pairs, either alternating (AB or BA) or non-alternating (AA or BB). When each

lever was set to reward alternately, a lever selection was defined as 5 or more presses on a lever. When both levers were set to reward, a lever selection was defined as the lever on which reinforcement was obtained.

Although the above analysis was conducted for each experiment, in many cases the outcome proved to be non-significant and therefore, in the presentation and discussion of some experiments reference is not made to statistical significance. However, in view of the strict training criterion employed in these experiments (Section 2.3.d, Procedure 2B), the occurrence of lever selections on the application of a stimulus which result in a deviation from an alternation of lever selection response is unlikely to reflect a random event.

The data is presented in the form of cumulative records as demonstrated in Fig. 2.3. The records were compiled using a graphics program (SigmaPlot, Jandel Scientific). The time scale is represented by the horizontal axis and the vertical axis scale corresponds to the number of lever presses. Each open symbol on the record represents a lever press and each downward line a reinforcement. The slope of the line joining the open symbols reflects the rate of lever pressing. In some instances, animals may have been rewarded after less than 20 lever presses. This situation arose due to 1 or 2 lever presses accumulating by mechanical stimulation induced by the firing of the food hopper solenoid on the delivery of food. Such artefact presses were edited from the data and this accounts for the FR 18 or FR 19 sometimes encountered in the records.

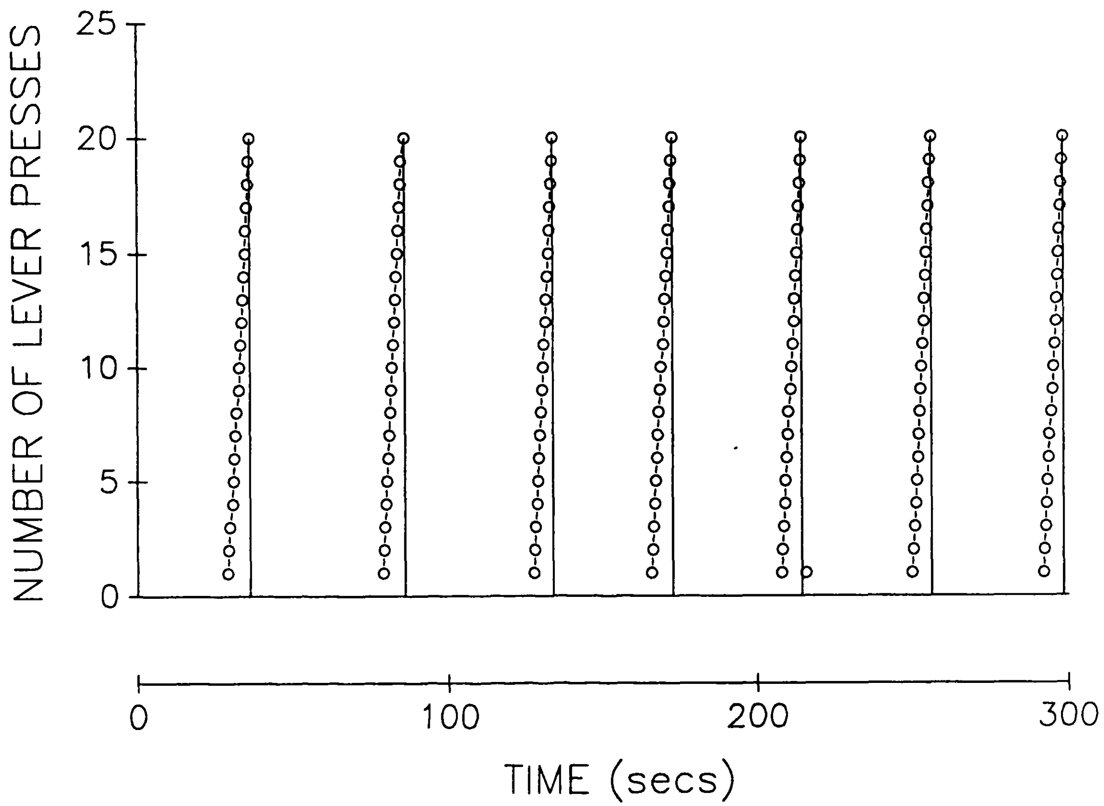


Fig. 2.3 An example of a cumulative record.

2.4 Methods used in the determination of whole brain concentrations of diazepam

2.4.a Preparation and extraction of mouse brain

Male mice and female mice at different stages of the oestrous cycle were treated with diazepam, as described in Section 2.2.a and then killed 60 mins later by cervical dislocation. Whole brains were removed immediately and placed on a glass plate on ice. The olfactory lobes were removed and the brains were then stored (for 3-11 weeks) in aluminium foil envelopes at -70°C.

Each sample was weighed while still frozen and then homogenised using a Polytron homogeniser (speed 10, 10 secs) in 5 volumes of ice cold HCl (0.1 M). Concentrated perchloric acid (72%,w/v) was then added to a concentration of 0.5 M and the homogenates left on ice for 10 mins to allow precipitation of proteins. Samples were centrifuged (28,000g; 10mins; 4°C) and the clear supernatant decanted into a fresh tube before addition of 2.5 volumes of 1 M $KHCO_3$ to neutralise the extract and precipitate the perchlorate. After a further 20 mins on ice, the extracts were centrifuged again and the supernatants decanted for storage overnight at -20°C.

To estimate the recovery of diazepam through the extraction procedure, known amounts of this drug (60-120 pmole) were added to brain homogenates. The protein content of the brain homogenates was assayed by the method of Bradford (1976) and standardised as BSA equivalents (Section 2.4.d).

2.4.b Radio-immunoassay

Concentrations of diazepam in the brain extracts were measured by inhibition of [^{3}H]-flunitrazepam binding to immunoglobulin G (IgG) class antibodies specific for the intact, pharmacologically active benzodiazepine nucleus (Fry et al., 1987).

Antiserum was prepared at a dilution of 1:7500 in Hepes/Tris/BSA/M.

Samples of brain extracts were added in triplicate to the antiserum, to a final total volume of 0.5 ml. The samples were vortexed and incubated overnight at 4°C. Tubes containing a range of known diazepam concentrations ($1x10^{-5}$-$1x10^{-12}$ M) were included in each assay for the construction of a standard curve. Blank controls were also included in each assay which contained buffer without antisera. The [^{3}H]-flunitrazepam was added to a concentration of 0.2nM, followed by a further incubation for at least 2 hrs at room temperature. To adsorb the IgG component of the serum prewashed Staphylococcus aureus cells (50ul at a final concentration of 0.01% v/v; Section 2.4.c) were incubated in excess with the samples. The incubation was terminated by vacuum filtration. After each vial was vortexed, ice cold PBS (4°C) was added and the contents were filtered immediately through prewashed (PBS at 4°C) glass fibre filters using a cell harvester (Brandel M-24R). The vials were then rinsed with PBS and the contents filtered. Radioactivity bound to the antibodies and trapped on the filters by the Staphylococcus aureus cells was measured by scintillation counting (Section 2.4.e).

2.4.c Preparation of Staphylococcus aureus cells

Formalin-fixed heat treated Staphylococcus aureus cells prepared as described by Kessler (1975) and stored (4°C) at a concentration of 10%, were washed prior to use as follows: aliquots were centrifuged (Beckman microfuge 12) at room temperature for 1 min at 12,000g, to sediment the cells. The supernatant was discarded by suction and the pellet resuspended to the original volume in PBS/BSA/M. When the cells were washed three times the required volume was removed and the remainder stored in PBS/BSA/M at 4°C.

2.4.d Protein assay: Dye binding method

The protein content of samples was measured according to the method of Bradford (1976) using the dye coomassie brilliant blue G. Binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 nm to 595 nm and it is the increase in absorption at 595 nm which is measured. For 2 litres of the dye solution, 150 mg of coomassie brilliant blue was

dissolved in 150 ml of ethyl alcohol (95% w/v) and 240 ml of phosphoric acid (85% w/v). The solution was diluted to 2 litres with distilled water and filtered when cool.

Samples (50µl) were added to quadruplicate test tubes containing 100µl of distilled water. Corresponding blanks contained the same volume of either distilled water or NaOH buffer. The fourth tube in each row contained a 50µl standard of BSA (25 µg). Coomassie brilliant blue solution (5 ml) was then added to all the tubes and the protein content estimated within 40 mins by measuring the absorbance at 595 nm in a spectrophotometer (Pye Unicam SP6 UV/VIS). After subtraction of reagent blanks, the protein concentrations (µg/ul) were calculated from the equation:

$$\frac{\text{mean sample absorbance (595nm)}}{\text{mean standard absorbance (595nm)}} \times \frac{\text{concentration of standard }(\mu g)}{\text{volume of sample }(\mu l)}$$

2.4.e Liquid scintillation counting

The glass fibre filters from filtration assays were transferred to 5 ml scintillation vials. Xylene-based scintillation fluid containing detergent (Optiphase X; 3 ml) was added to the samples. The vials were then vortexed and the radioactive content measured by counting for 10 mins in a Tri-Carb liquid scintillation spectrometer. Each sample was counted in two channels, one of which included an integral external standard (^{226} Radium). To correct for the variation in counting efficiency of individual samples, channel ratio quench correction curves were constructed by calibration using a tritium standard of n-hexadecane.

2.4.f Data analysis

Radio-immunoassay data expressed as dpm representing the displacement of [^{3}H]-flunitrazepam by known concentrations of diazepam were transformed by the Hill equation (Hill,1910) into linear plots of log%B / 100-%B vs log [D] , where [D] = the displacer concentration and %B = percent of control

binding. A computerised linear least squares method (with 95% confidence limits) was used to fit Hill plots and estimate the sample concentrations by comparison with known concentrations of diazepam, in their ability to displace [^{3}H]-flunitrazepam. The amounts of diazepam extracted from the different brain samples were expressed as pmole/gm wet tissue weight.

A Kruskal-Wallis one way analysis of variance was used to test for any significant change in diazepam concentration in female mouse brain during the oestrous cycle. Diazepam concentrations from male and female mouse brain were compared for statistical difference using the Students t-test (two-tailed).

Chapter 3

An investigation of ovarian cycle influence on $GABA_A$/benzodiazepine receptor function

3.1 Introduction

3.2 Results and discussion

3.2.a Effect of diazepam on the number of light/dark transitions made by male mice tested in the light/dark box.

3.2.b Behaviour of untreated and diazepam treated female mice at different stages of the oestrous cycle tested in the light/dark box.

3.2.c Effect of diazepam on four parameters of the behaviour of female mice at metoestrus I tested in the light/dark box.

3.2.d Measurements of whole brain concentrations of diazepam in male mice and in female mice at different stages of the oestrous cycle.

3.2.e Physiological mechanisms which could account for the observed fluctuations in response to diazepam during the oestrous cycle.

3.3 Conclusions

3.1 Introduction

Hormonal changes that occur during the ovarian cycle in women are known to influence a variety of clinical disorders (see Magos & Studd, 1985; Maggi & Perez, 1985). Disorders of presumed central nervous system (CNS) origin include the increased frequency of seizures seen in epileptic patients in the immediate pre-menstrual and menstrual phase (catamenial epilepsy; Gowers, 1881; Ansell & Clarke, 1956; Laidlaw, 1956; Holmes, 1988). The premenstrual phase can also be associated with a dysphoria which, if severe, is manifest as the so-called pre-menstrual syndrome, during which some women are reported to increase their consumption of minor tranquillising drugs, alcohol or other central depressants (Belfer et al., 1971; Reid & Yen, 1981). Animal studies also support a role of ovarian steroid hormones in the modulation of seizure threshold and emotional state (see Maggi & Perez, 1985; Rodriguez-Sierra et al., 1984).

A possible site of action for the above effects of ovarian steroids on seizure thresholds and emotionality is provided by the $GABA_A$/benzodiazepine receptor complex. Certain sedative metabolites of progesterone are known to have direct actions on this receptor complex at the level of the neuronal membrane (see Chapter 1, Section 1.3.b). They have been shown to enhance the action of the $GABA_A$ receptor agonist muscimol, on slices of rat cuneate nucleus in vitro (Harrison & Simmonds, 1984) and to prolong inhibitory postsynaptic currents mediated by $GABA_A$ receptors in cultures of rat hippocampus (Harrison et al., 1987). Further allosteric effects of these progesterone metabolites on the $GABA_A$ receptor complex are indicated by their ability to increase the affinity of specific benzodiazepine receptor binding in rat brain membranes (Majewska et al., 1986). These metabolites are produced from the ovary of the rat and their secretion like that of progesterone is subject to fluctuation during the ovarian cycle (Holzbauer, 1971, 1975; Ichikawa et al., 1974). Such metabolites are also produced in the pituitary, hypothalamus and other brain regions (Cheng & Karavolas, 1973; Rommerts & Van der Molen, 1971; Robinson & Karavolas, 1973; Krause & Karavolas, 1980; Hanukoglu et al.,

1977).

Other actions of ovarian hormones on the $GABA_A$ receptor also appear to occur. Administration of oestradiol to ovariectomised rats has been reported to decrease binding of the $GABA_A$ receptor agonist [^{3}H]-muscimol to high affinity sites in specific oestrogen-sensitive areas of the hypothalamus, amygdala and midbrain, changes which are reversed upon the subsequent administration of progesterone (Schumacher et al., 1989a). The same authors have shown that treatment of ovariectomised rats with oestradiol followed by progesterone increases muscimol binding in area CA1 of the hippocampus (Schumacher et al., 1989b) and others find such hormone treatments to increase muscimol binding in additional forebrain areas (Maggi & Perez, 1984). Specific [^{3}H]-benzodiazepine binding is also affected by these steroid hormones. Administration of oestradiol to ovariectomised rats has been shown to affect [^{3}H]-flunitrazepam binding in hypothalamic and amygdaloid areas. Subsequent treatment with progesterone reversed these changes and enhanced binding in the baso-lateral amygdala and in area CA1 of the hippocampus (Canonaco et al., 1989). These latter effects were dependent on a high dose of progesterone and could be mimicked by in vitro treatment of the particular brain regions with the progesterone metabolite 3α-hydroxy-5α-pregnan-20-one.

From the above it can be seen that there is considerable evidence to implicate a role for the ovarian steroid hormones and their metabolites in the modulation of $GABA_A$ receptor function. However these studies are based on measures of $GABA_A$ receptor function in vitro and/or the exogenous administration of steroids to ovariectomised animals. Hence it is difficult to interpret these results in terms of the interactions which may occur between the ovarian steroids, steroid metabolites and the $GABA_A$/benzodiazepine receptor complex, in the intact animal in vivo. The aim of the present study was to evaluate the significance and possible mode of action of ovarian steroid hormones on $GABA_A$/benzodiazepine receptor function, by using the light/dark box test of exploration to monitor the fearfulness of mice and their sensitivity to a presumed anxiolytic action of

diazepam at different stages of the oestrous cycle.

3.2 Results and discussion

3.2.a Effect of diazepam on the number of light/dark transitions made by male mice tested in the light/dark box

In male mice, injection of diazepam (0.2-2.0 mg/kg, i.p., 60 min) caused a dose dependent increase in the number of transitions between the light and dark chambers of the exploratory apparatus (Fig. 3.1). This result is in agreement with that previously reported by Crawley (1981). However, in the present study, injection of the drug vehicle alone to naive mice caused a significant ($P < 0.05$) decrease in the number of light/dark transitions, in comparison to untreated mice. This did not appear to be due to the drug vehicle itself, because similar decreases were seen in mice injected with the same volume of phosphate-buffered saline or mice given a sham injection (Fig. 3.2 (a)). Such an effect of the intraperitoneal injection procedure was not seen in male mice that had been handled daily for the preceding 14 days (Fig. 3.2 (b)); a degree of handling comparable to that experienced by female mice in the sampling of vaginal smears and which did not show a vehicle effect at most stages of the cycle (see below). Therefore these results suggest that daily handling renders male mice less susceptible to the acute stressful effect of an intraperitoneal injection.

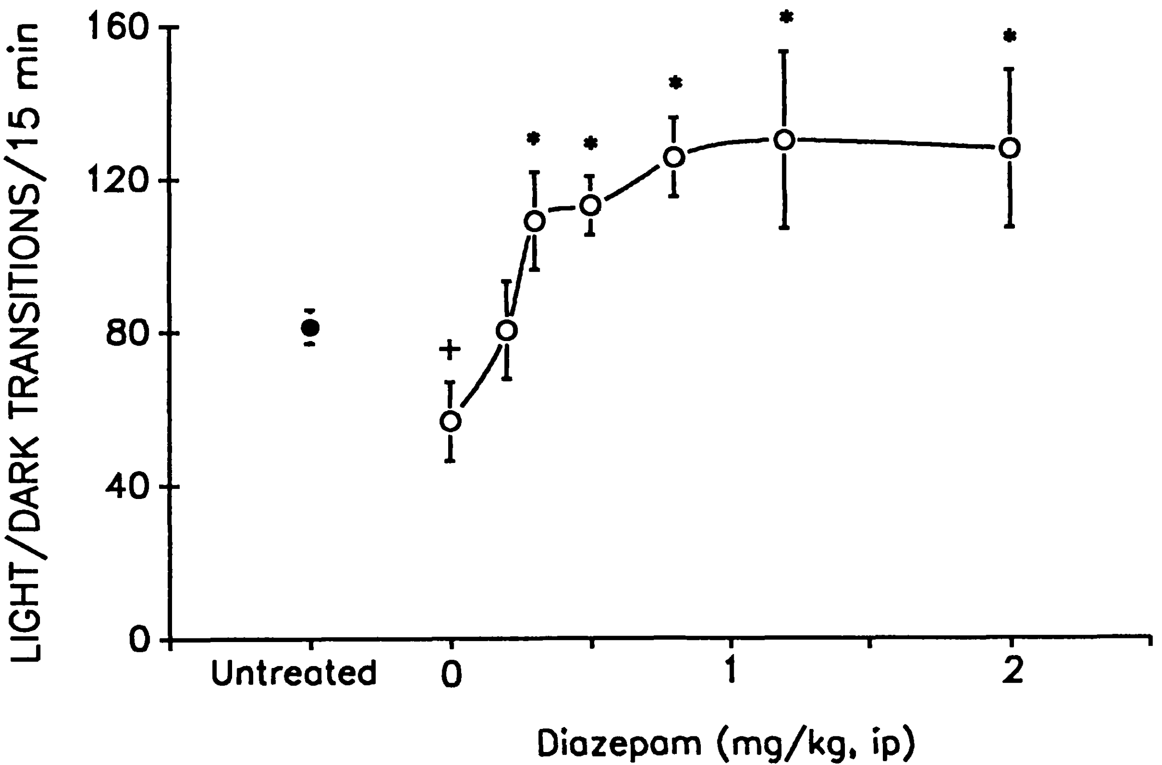


Fig. 3.1 Effect of Diazepam (0.2-2.0 mg/kg,i.p.) on the number of transitions made by male mice between the light and dark chambers of the light/dark box. Mice were injected with diazepam or the drug vehicle alone 60 min before the test, which lasted for 15 min and was initiated by placing the mouse in the dark chamber. Each mouse was tested only once and experiments included untreated mice for comparison. Points are mean ± S.E.M. ($n \geq 6$). *$P<0.05$ v vehicle alone, +$P<0.05$ v untreated; Mann-Whitney U test.

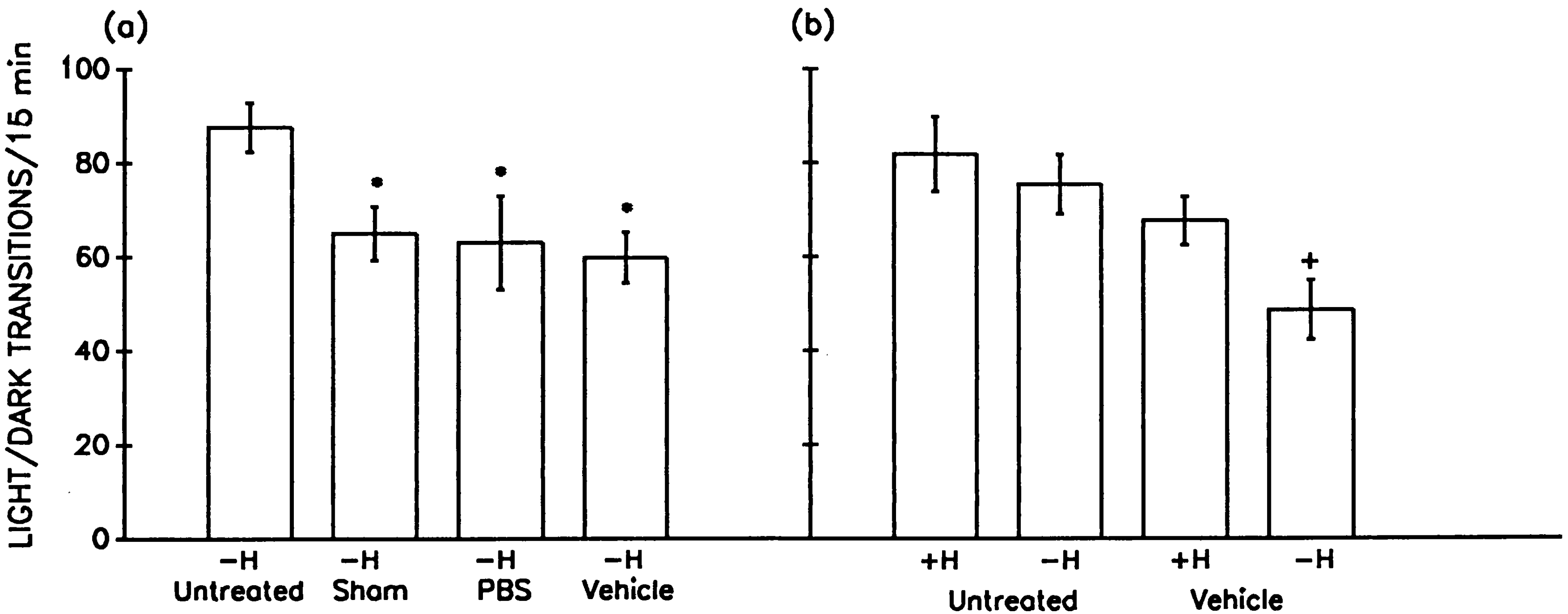


Fig. 3.2 Effect of a sham intraperitoneal injection , an injection of phosphate buffered saline (PBS) or an injection of the diazepam vehicle on the number of light/dark transitions made in the light/dark box by male mice that were a) naive (-H) and b) naive (-H) or handled (+H) daily for 14 days before the test. Values are mean ± S.E.M. ($n \geq 9$). *$P<0.05$ **v** untreated , +$P<0.05$ **v** handled and vehicle treated ; Mann-Whitney U test.

3.2.b Behaviour of untreated and diazepam treated female mice at different stages of the oestrous cycle tested in the light/dark box

From the diazepam dose response relation observed in male mice (Section 3.2.a), a dose of diazepam (0.28 mg/kg) that caused an increase in light/dark transitions to about 50% of maximum was chosen to test the sensitivity of female mice to the drug at different stages of the oestrous cycle. Untreated mice at each cycle stage were also tested to investigate possible fluctuations in fearfulness during the cycle.

Effect of oestrous cycle stage on the number of light/dark transitions made by untreated female mice

During the oestrous cycle, no significant fluctuations were found in the number of light/dark transitions made by untreated female mice ($P > 0.05$; Fig. 3.3). The ovarian secretion of the ring A reduced pregnane derivatives, which have been shown to enhance GABAergic function in vitro (see Chapter 1, Section 1.3.b), is greatest from late pro-oestrus to metoestrus (Holzbauer, 1975; Ichikawa et al., 1974). Since a facilitation of light/dark transitions during oestrus or metoestrus was not observed in the present study, these results suggest that the ovarian progesterone metabolites do not exert any physiological influence on the anxiety state of the animal, as reflected by a shift in a light/dark conflict. Others have reported an increased exploration of novel environments (Gray & Levine, 1964; Burke & Broadhurst, 1966; Gray & Cooney, 1982) and decreased conditioned avoidance behaviour (Gray, 1977; Rodriguez-Sierra et al., 1984) of rats and mice at oestrus; behaviour that would be consistent with a decreased anxiety state and would serve the biological function of facilitating mate selection. However, such changes in behaviour at oestrus may not be due to a direct action of progesterone metabolites at the level of the neuronal membrane, because progesterone-induced decreases in conditioned avoidance behaviour in the rat are only seen after the prior administration of oestrogens (Rodriguez-Sierra et al., 1984). Some authors have

attributed the increased exploratory behaviour of oestrus rats to general metabolic changes in feeding and locomotor activity, rather than decreased fearfulness (Drewett, 1973; Birke & Archer, 1975). Such non-specific increases in locomotor activity were not apparent in the present study or in previous studies of mice (Gray, 1978; Gray & Cooney, 1982) which indicated a specific increase in the exploration of the brightly lit portion of a novel environment at oestrus. There was no comparable increase in the number of light/dark transitions made by oestrus mice in the present test apparatus although there was a (non-significant) fall in the number of transitions made by mice at dioestrus, the stage of the ovarian cycle which the above authors chose to compare with oestrus.

Effect of the drug vehicle alone on the number of light/dark transitions made by female mice

Injection of female mice with the drug vehicle alone caused a decrease in the number of transitions at late dioestrus and pro-oestrus but no significant effect of injection with the drug vehicle alone was observed at any other stage of the cycle (Fig. 3.3). The effect of an intraperitoneal injection in male mice was no longer seen when the mice were handled for a period of 14 days before the test (Section 3.2.a; Fig. 3.2 (b)). Male mice were subjected to this period of handling in an attempt to mimic the handling experienced by female mice in the sampling of vaginal smears. Therefore, these results suggest that daily handling of male or female mice renders them less susceptible to the acute stressful effects of an intraperitoneal injection, as revealed by their subsequent performance in the light/dark box test apparatus, but that at late dioestrus or pro-oestrus in female mice, other mechanisms appear to intervene which influence this adaptation to stress. One such mechanism might be the release of adrenocorticotropic hormone (ACTH). Both corticotropic releasing factor (CRF) and ACTH have apparent anxiogenic effects in the rat (File & Vellucci, 1978; Dunn & File, 1987) and the release of ACTH appears to be responsible for the decreased exploratory behaviour of mice following acute restraint stress (Berridge & Dunn, 1987; Haas & George, 1988). Moreover, the release of ACTH is known to fluctuate during the oestrous cycle, with peak plasma

concentrations at pro-oestrus (Buckingham et al., 1978). An increase in CRF and ACTH release, from late dioestrus to pro-oestrus could therefore have rendered the female mice at these stages of the cycle more susceptible to the acute stressful effects of the intraperitoneal injection procedure and account for the present observations.

Effect of diazepam on the number of light/dark transitions made by female mice at different stages of the oestrous cycle.

Diazepam at 0.28 mg/kg increased the number of transitions when given to female mice at oestrus and dioestrus ($P < 0.05$) but had no significant effect at late dioestrus, pro-oestrus or metoestrus II (Fig. 3.3). At metoestrus 1 this test dose of diazepam significantly ($P < 0.05$) decreased the number of light/dark transitions (Fig. 3.3).

Although diazepam (0.28 mg/kg) increased the number of light/dark transitions made by male mice (Fig. 3.1) and female mice at oestrus and dioestrus (Fig. 3.3), there was no drug induced change in other test parameters such as the latency to emerge from dark chamber or the time and rearing activity in the light chamber (data not shown). Such changes might be expected to occur in response to an anxiolytic drug action. Therefore, these observations question the validity of the increase in light/dark transitions as an index of anxiety and hence the failure to observe a change in this parameter in untreated mice during the cycle may reflect the insensitivity of this test to the detection of a change in fearfulness. This problem is addressed in the following Chapter. However, a change in the number of light/dark transitions has provided a measure of diazepam responsiveness in male mice and in female mice during the oestrous cycle. In an attempt to account for the observed fluctuations in response to this drug during the oestrous cycle, further experiments were performed and the results obtained are described in the following sections.

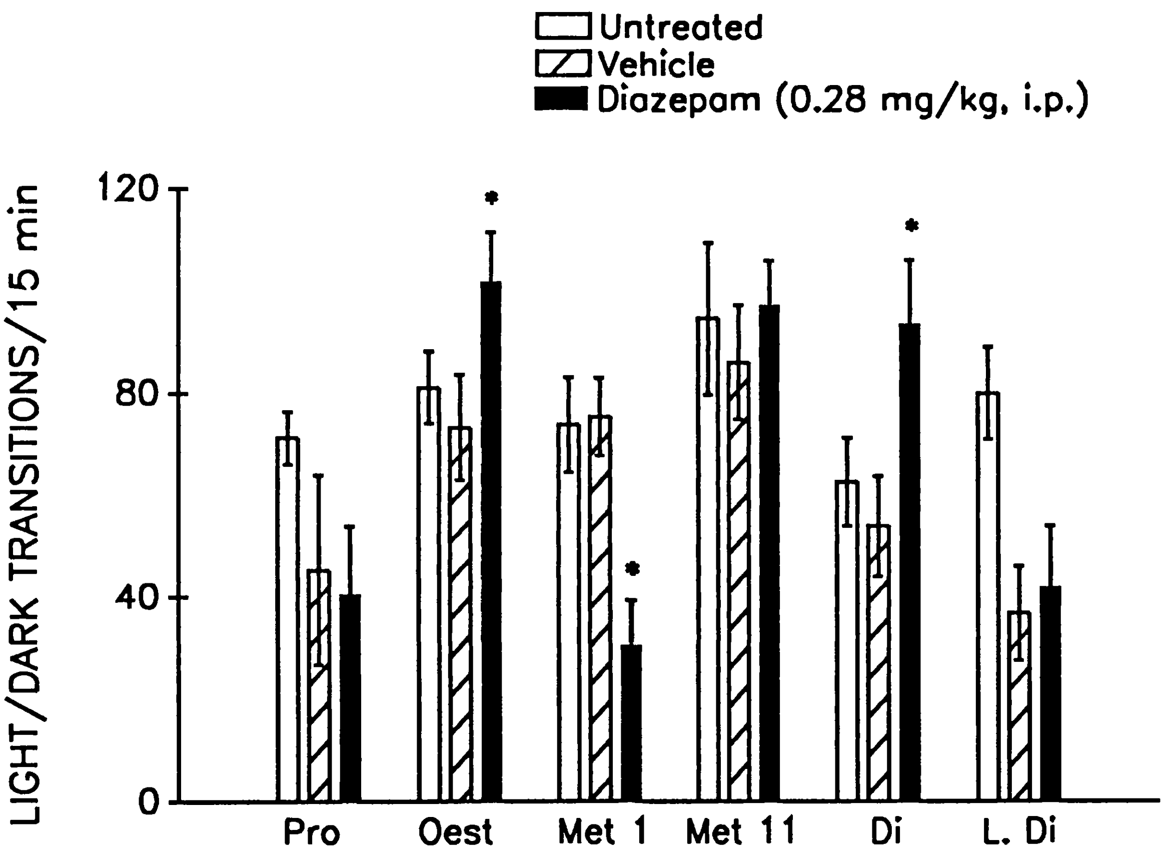


Fig. 3.3 Transitions between light and dark compartments of the light/dark box made by female mice at different stages of the oestrous cycle : pro-oestrus (Pro) , oestrus (Oest) , metoestrus 1 (Met 1) , metoestrus 11 (Met 11) , dioestrus (Di) and late dioestrus (L. Di). Mice were tested 60 min after injection of diazepam (0.28 mg/kg,i.p.) , after injection of the drug vehicle alone or without any prior treatment. The values shown are mean ± S.E.M. ($n \geq 6$). *$P<0.05$ **v** drug vehicle alone ; Mann-Whitney U test.

3.2.c Effect of diazepam on four parameters of the behaviour of female mice at metoestrus 1 tested in the light/dark box

Fig. 3.4 compares the effects of diazepam at 0.28 and 1.0 mg/kg on four parameters of the behaviour of female mice at metoestrus 1, tested in the light/dark box. The lower dose of 0.28 mg/kg diazepam not only decreased the number of light/dark transitions (Fig. 3.4 (a)) but also caused a significant ($P < 0.02$) decrease in the time spent in the light chamber (Fig. 3.4 (c)) and in the number of rearings made in this chamber (Fig. 3.4 (d)), while the latency to emerge from dark to light chamber was significantly ($P< 0.05$) increased (Fig. 3.4 (b)). The higher dose of diazepam produced a significant ($P < 0.05$) increase in the number of light/dark transitions (Fig. 3.4 (a)) but no change in the other three parameters measured (Fig. 3.4 (b-d)). This increase in transitions was comparable to that seen upon administration of the lower dose of the drug to male mice (Fig. 3.1) or to female mice at oestrus or dioestrus (Fig. 3.3). Therefore the decrease in transitions at metoestrus 1 in response to 0.28 mg/kg diazepam could not have been due to these animals being supersensitive to the drug and displaying the sedative actions normally seen at higher doses (Crawley, 1981). Rather, these results suggest that female mice tested after treatment with the lower dose of 0.28 mg/kg diazepam at metoestrus 1 display an increased fearfulness and/or photophobia, as reflected by an increased latency to enter the light chamber and a reduction of rearing activity and time spent in the light chamber. Such a decrease in light/dark transitions, with a corresponding decrease in the time in the light chamber of a similar exploratory apparatus, has been reported following administration of the anxiogenic ß-carboline ß-CCM to male mice (Belzung et al., 1987).

If mice at metoestrus 1 are subsensitive to diazepam, then the possibility arises that their apparent anxiogenic response to the lower 0.28 mg/kg dose of this drug represents a general response of mice to low benzodiazepine receptor occupancy or efficacy in the brain. This does not appear to be the case, because administration of diazepam to previously handled male mice at a range of doses (0.025-0.1 mg/kg), too low to provoke an increase in light/dark transitions fails to induce a decrease in the number of transitions or to change the latency

to emerge, time in the light chamber or rears in the light chamber (Fig 3.5). Two previous reports of administration of a low dose of diazepam (0.1-0.3 mg/kg) to male rats have revealed a drug induced decrease in ambulation around an open field arena and a suppression of fluid intake. These effects were specific to the low doses of the drug and are inconsistent with the expected behavioural profile of benzodiazepine agonists (Cooper, 1983, 1985b). In the present study, the decrease in light/dark transitions and increase in apparent fearfulness seen upon injection of female mice with diazepam at metoestrus 1, represents an anomalous response to the drug that appears to be unique to female animals at this stage of the ovarian cycle.

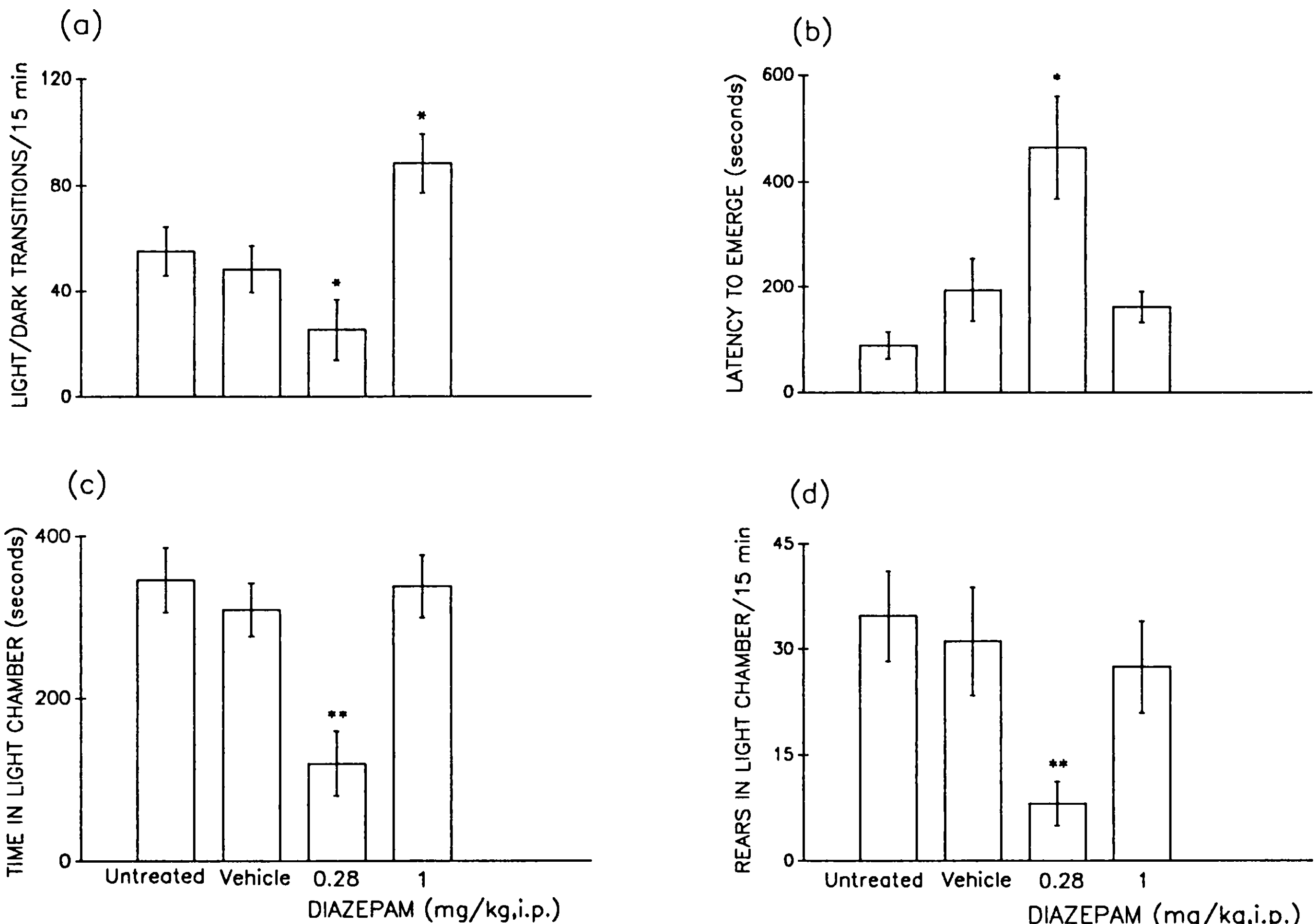


Fig. 3.4 Comparison of the effect of diazepam at 0.28 and 1.0 mg/kg , i.p. on four parameters of the behaviour of female mice tested in the light/dark box at metoestrus 1 : (a) number of light/dark transitions , (b) latency to emerge from dark chamber , (c) time spent in light chamber and (d) number of rears made in light chamber. All values mean ± S.E.M. ($n \geq 10$). * $P < 0.05$, ** $P < 0.02$ v drug vehicle alone ; Mann-Whitney U test.

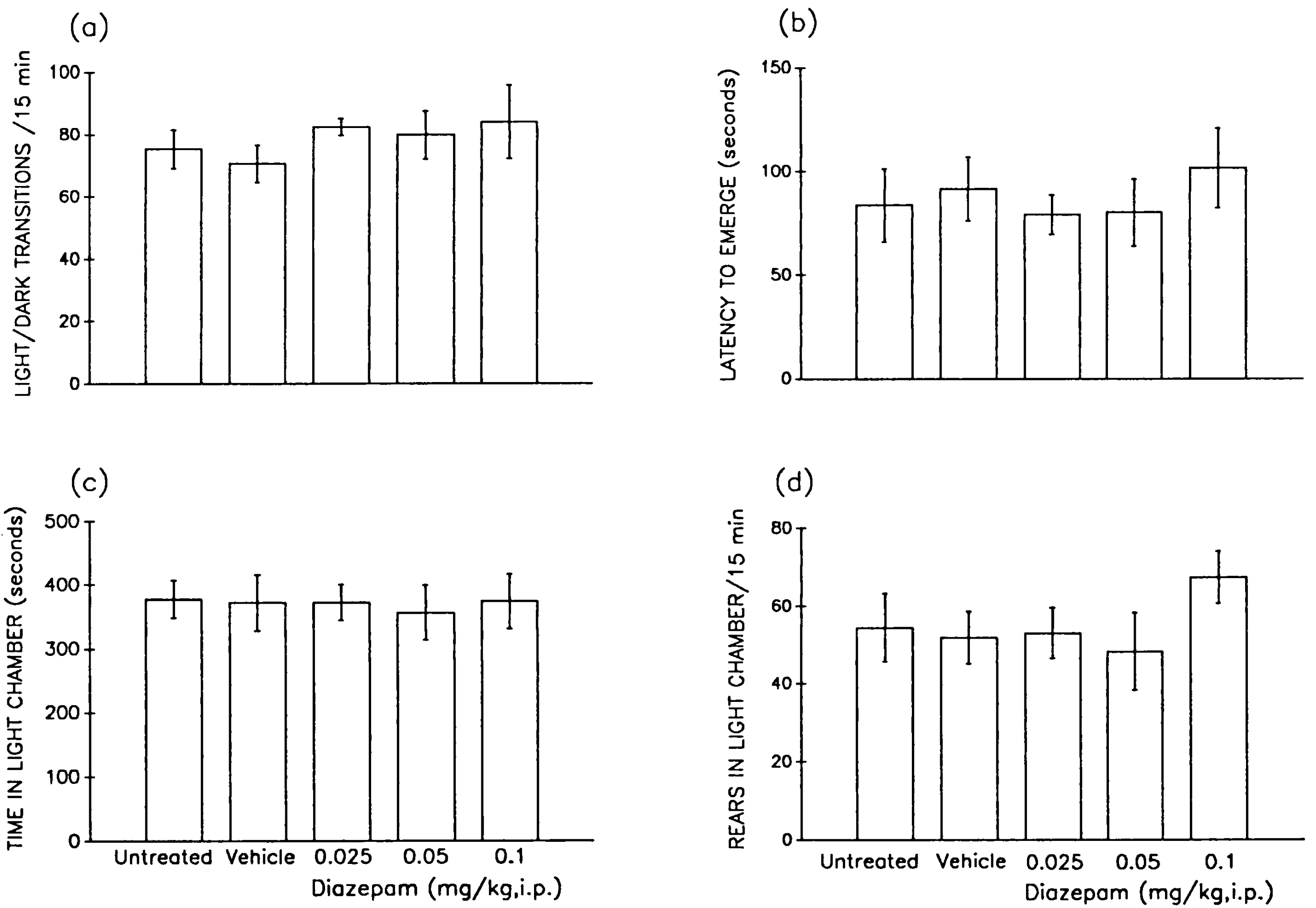


Fig. 3.5 Effect of diazepam (0.025-0.1 mg/kg,i.p.) on four parameters of behaviour of male mice that were handled for 14 days and tested in the light/dark box : (a) number of light/dark transitions , (b) latency to emerge from dark chamber , (c) time spent in light chamber and (d) number of rears made in light chamber. All values mean ± S.E.M. (n = 6).

3.2.d Measurements of whole brain concentrations of diazepam in male mice and in female mice at different stages of the oestrous cycle

The whole brain concentrations of diazepam (or its active metabolites) 60 min after the intraperitoneal injection of this drug (0.28 mg/kg) to male mice or to female mice at different stages of the oestrous cycle are shown in Table 3.1 . Concentrations of benzodiazepine were significantly lower in female than in male mouse brain ($P < 0.02$) but no significant fluctuations were seen during the oestrous cycle ($P > 0.05$). Therefore the changes in response to diazepam during the oestrous cycle shown in Figs. 3.3 and 3.4, do not appear to be due to variations in the amount of this drug or of its active metabolites, reaching the specific neuronal benzodiazepine receptors in the brain. In addition to diazepam the antibodies used in the immuno-assays would also recognise the three major metabolites of diazepam found in brain, namely N-desmethyldiazepam, oxazepam and 3-hydroxydiazepam (Fry et al.,1987). Two of these metabolites, N-desmethyldiazepam and oxazepam have been found to account for most of the benzodiazepine accumulating in the brain of the mouse 60 mins after the oral administration of diazepam (Coutinho et al., 1970). They have receptor binding affinities and pharmacological potencies comparable to diazepam itself (Randall et al., 1974; Haefely et al.,1985) and presumably account for the prolonged action of this drug in the mouse. Although concentrations of benzodiazepines extracted from the female mouse brain after intraperitoneal injection of diazepam did not fluctuate during the oestrous cycle, they were significantly lower than those recovered from male mice. In this respect, the mouse may be similar to man and have a faster metabolic clearance of diazepam in the female (Greenblatt et al., 1980).

Table 3.1

Whole brain concentrations of diazepam and its pharmacologically active metabolites 60 min after injection of the drug at 0.28 mg/kg , i.p. to male mice and to female mice at different stages of the oestrous cycle. All values mean ± S.E.M. ($n \geq 8$). * $P < 0.02$ v females ; Student's t-test.

Sex	Whole brain diazepam equivalents (pmol/gm wet weight)
Male	106.4 ± 9.9*
Female	
pro-oestrus	82.2 ± 9.4
oestrus	67.9 ± 10.5
metoestrus I	88.3 ± 8.2
metoestrus II	83.1 ± 8.3
dioestrus	87.6 ± 10.9

3.2.e Physiological mechanisms which could account for the observed fluctuations in response to diazepam during the oestrous cycle

Although ovarian progesterone metabolites have reported enhancing effects on GABAergic function in vitro and are able to increase the affinity of specific benzodiazepine receptor binding in rat brain membranes (see Chapter 1, Section 1.3.b), a role for these metabolites in producing the observations of the present study is precluded since their pattern of secretion is highest from late pro-oestrus to metoestrus (Holzbauer, 1975; Ichikawa et al., 1974) but the highest sensitivities to diazepam were found at oestrus and dioestrus and not in the intervening metoestrus stages. However, since the two stages of oestrus and dioestrus are preceded by an increased production of progesterone (Butcher et al., 1974 ; Nelson et al., 1981) and since this steroid can be metabolised by brain tissue (Rommerts & Van der Molen, 1971; Cheng & Karavolas, 1973; Robinson & Karavolas, 1973; Krause & Karavolas, 1980; Hanukoglu et al.,

1977), it is possible that benzodiazepine sensitivity in the CNS is enhanced at these stages by the localised conversion of progesterone to its active metabolites. Such metabolites have been shown to have progesterone-like effects on neuroendocrine processes such as gonadotropin secretion (Brann et al., 1990) and lordotic behaviour (Czaza et al., 1974).

Other possible physiological actions of ovarian steroid hormones that could account for the behavioural fluctuations in sensitivity to diazepam during the oestrous cycle might be mediated by oestrogen or progesterone receptors regulating gene expression in the CNS. Pretreatment for 24 hours with low doses of oestradiol has been reported to attenuate diazepam sensitivity in the ovariectomised rat (Nomikos & Spyraki, 1988). Although this action of oestradiol might explain the present observations of decreased diazepam sensitivity at late dioestrus and pro-oestrus when plasma oestrogens are at their peak (Butcher et al., 1974; Nelson et al., 1981) it is unlikely to explain the decreased sensitivity to diazepam seen at metoestrus. A more plausible explanation would be the modulation of CNS benzodiazepine sensitivity by progesterone receptors operating through the genome. This explanation deserves serious consideration because the two ovarian cycle stages, oestrus and dioestrus, at which the highest sensitivity to diazepam was observed (Fig. 3.3) are the only stages preceded by an increased production of progesterone (Butcher et al., 1974; Nelson et al., 1981). In the female rat, increases in the concentration of plasma progesterone are known to be followed by an increase in the number of progesterone receptors found within cell nuclei in the brain (Rainbow et al., 1982). Progesterone actions at oestrogen-induced receptors in the brain are well known to induce expression of genes facilitating and terminating sexual receptivity and oestrous behaviour in rodents (see Ganten & Pfaff, 1985) and to reduce conditioned avoidance behaviour in the rat (Rodriguez-Sierra et al., 1984). Although certain ring A reduced metabolites of progesterone have been shown to produce progesterone-like effects on some neuroendocrine processes as described above, these processes can be inhibited in the intact animal by a specific antagonism of the progesterone receptor (Rao & Mahesh, 1986), which itself does not bind these metabolites (Gee et al., 1988) or by specific inhibitors of RNA or protein synthesis (see Ganten & Pfaff, 1985).

Therefore mediation of neuroendocrine processes by metabolites of progesterone may be dependent on an initial genomic action of progesterone. For instance, progesterone could regulate the expression of its metabolic enzymes or other factors related to their activity. Such a genomic action of progesterone could also account for an alteration in the CNS sensitivity of female mice to diazepam. Evidence exists which indicates an ovarian regulation of central progesterone metabolism. The conversion of [^{3}H]-progesterone to the sedative ring A reduced metabolites and the activity of the enzymes responsible have been shown to fluctuate in the rat hypothalamus during the oestrous cycle (Cheng & Karavalos, 1973; Krause et al., 1981). However, for the purpose of the present study, the results presented by these authors are difficult to interpret since only one time point was monitored at each cycle stage.

As both the induction of sexual receptivity and the control of luteinising hormone release by ovarian steroids appear to involve GABAergic mechanisms in the hypothalamus (Mansky et al., 1982; Mascó et al., 1986; Donoso et al., 1986) complex interactions might be expected to exist between the ovarian steroid hormone receptor systems and the $GABA_A$/benzodiazepine receptor complex during the oestrous cycle. Administration of oestrogen and/or progesterone to ovariectomised rodents affects both GABA and benzodiazepine binding (see Section 3.1). Such changes and/or the decreases which treatment with oestradiol or progesterone induces in the GABA synthetic enzyme glutamate decarboxylase (GAD; Wallis & Luttge, 1980) in certain brain areas, in addition to possible changes in central progesterone metabolising enzymes, may contribute to fluctuations in response to diazepam observed during the oestrous cycle in the present experiments.

3.3 Conclusions

As described previously (Crawley, 1981), intraperitoneal injection of diazepam caused a dose dependent increase in the number of transitions made by male mice between the light and dark chambers of a novel environment. When female mice were tested with the dose of diazepam which caused approximately 50% of the maximum increase in light/dark transitions in male mice, a diazepam response was evident only in mice at oestrus and dioestrus. Mice at pro-oestrus, late dioestrus and metoestrus II showed no response to diazepam while this dose of the drug produced a decrease in transitions in mice tested at metoestrus 1.

Further experiments revealed that this effect of diazepam at metoestrus 1 was not due to a sedative action of the drug but rather reflected a response of increased fearfulness or photophobia. The behavioural fluctuations in response to diazepam observed during the oestrous cycle could not be attributed to a change in drug metabolism or distribution since no significant differences were found in the whole brain concentrations of benzodiazepine-like immunoreactivity extracted from the brains of mice injected with diazepam at different stages of the cycle.

The results presented preclude a role of the depressant progesterone metabolites of ovarian origin in exerting a physiological influence on the anxiety state of the animal or in the modulation of CNS sensitivity to diazepam, since behavioural fluctuations were not observed in untreated female mice during the cycle and the pattern of ovarian secretion of these metabolites does not coincide with the stages at which highest sensitivity to diazepam was observed. However, the failure to detect a change in the anxiety state of untreated female mice during the oestrous cycle may be because the increase in light/dark transitions in this apparatus, does not provide an accurate measure of exploration or anxiolytic activity.

A possible explanation for the observed fluctuations in response to diazepam during the oestrous cycle may be the modulation of CNS

benzodiazepine sensitivity by a steroidal regulation of the expression of certain gene products which influence $GABA_A$/benzodiazepine synaptic function. Since the two oestrous cycle stages, oestrus and dioestrus, at which the highest sensitivity to diazepam was observed, are the only stages preceded by an increased production of progesterone (Butcher et al., 1974; Nelson et al., 1981) a genomic action of this steroid may account for the observed diazepam responses.

However, further explanation must await a study of the effects of specific ovarian steroid hormones on the expression of genes for the $GABA_A$/benzodiazepine receptor subunits, the enzyme GAD and the progesterone metabolising enzymes in the brain. Steroidal regulation of such neurochemical events, which underlie the present observation of changes in sensitivity to diazepam, may be responsible for the changes in epileptic seizure frequency, mood and consumption of central depressant drugs that sometimes occur during the ovarian cycle.

Chapter 4

An evaluation of the light/dark choice test of exploration as a model of anxiety in the mouse.

4.1 Introduction

4.2 Results and discussion

4.2.a Effects of diazepam or d-amphetamine on the behaviour of male mice tested in the light/dark box

4.2.b Attempts to distinguish effects of diazepam on exploratory behaviour from non-specific locomotor behaviour in the light/dark box apparatus

4.2.c The contribution of a light/dark conflict to the changes detected in response to diazepam in the light/dark box apparatus

4.2.d Habituation of male mice to the light/dark box apparatus and the effects of diazepam or d-amphetamine treatment on habituated animals

4.3 Conclusions

4.1 Introduction

The light/dark choice test of exploration was first proposed as an animal model of anxiolytic drug action by Crawley and coworkers (Crawley & Goodwin, 1980; Crawley, 1981). This proposal was based on the finding that benzodiazepine tranquillisers and other drugs with anti-anxiety action such as meprobamate could induce a dose-related increase in one parameter of the behaviour of male mice tested in the apparatus. This behavioural parameter was the number of transitions made between the light and dark chambers of the test box. Such an increase in the number of light/dark transitions was reported to be specific for drugs with anti-anxiety properties since other compounds e.g. neuroleptics and anti-depressants, tested did not produce this change in behaviour (Crawley, 1981). A drug induced increase in the number of transitions was thought to reflect an increased exploration of the light chamber due to a reduction in a light/dark conflict (Crawley & Goodwin, 1980). The ability of several benzodiazepines to induce an increase in light/dark transitions was shown to correlate well with their known clinical anti-anxiety potencies (Crawley, 1981). The specificity of this parameter change to the anti-anxiety property of the benzodiazepines as opposed to their stimulant or sedative actions was confirmed by testing these compounds in a separate motor activity test (Crawley & Goodwin, 1980).

On the basis of the above reports, the light/dark box test appeared to offer a simple and rapid means of assessing the effects of various stimuli on the state of anxiety and of screening new compounds with putative anxiolytic activity. It was not subject to the disadvantages of several of the other animal tests described in Chapter 1 (Sections 1.2 and 1.5). For instance, it did not entail any treatments which could possibly interfere with the animal's motivational or sensory state and it did not require any animal training. However, when this test was employed to study the influence of the ovarian cycle on anxiety and $GABA_A$/benzodiazepine receptor function in the mouse (Chapter 3) some ambiguous results were obtained. Contrary to the findings of another author using a similar apparatus (Gray, 1978), an increase in exploratory behaviour was not observed in oestrus mice. Also, a diazepam

induced increase in the number of light/dark transitions was not accompanied by changes in the other test parameters (e.g. latency to emerge from the dark to light chamber) which would suggest increased exploration of the brightly lit chamber.

Hence, the aim of the present study was to evaluate the light/dark box test both behaviourally and pharmacologically to assess the validity of the behavioural parameters of this test as indices of anxiety and thereby provide a better understanding of the results obtained from the use of the test.

4.2 Results and discussion

4.2.a Effects of diazepam or d-amphetamine on the behaviour of male mice tested in the light/dark box.

The effects of diazepam on seven parameters of the behaviour of male mice tested in the light/dark box are shown in Fig. 4.1. In agreement with previous results (Chapter 3, Section 3.2.a), injection of diazepam (0.1-1.0 mg/kg, i.p.; 60 min) caused a significant ($P < 0.002$) increase in the number of light/dark transitions (Fig. 4.1.a). The significant ($P < 0.05$) decrease in this parameter in response to the drug vehicle alone is consistent with the previously reported finding of a decrease in transitions due to an acute stressful effect of the injection procedure rather than the drug vehicle itself (Chapter 3, Section 3.2.a).

Light chamber activity was increased significantly ($P < 0.05$) at the two higher doses of diazepam (0.5 and 1.0 mg/kg) tested (Fig. 4.1(b)) while dark chamber activity showed a significant ($P < 0.002$) increase on injection of 0.5 mg/kg diazepam (Fig. 4.1(c)). In contrast to the findings of Crawley (1981), a diazepam induced increase in chamber activity was not observed for each dose of the drug which produced a significant increase in the number of light/dark transitions. This author interprets such drug induced increases in locomotion in the apparatus as an indication of an increase in the animal's exploratory

tendencies which can be distinguished from a non-specific motor effect of the drug by measuring locomotion in independent groups of similarly treated mice in a bare undifferentiated cage (Crawley & Goodwin, 1980). However, this method of distinguishing the effect of a drug on exploration from non-specific locomotion is subject to two criticisms: (1) the effective stimulant or sedative dose of a drug can vary according to the test used (e.g. see File et al., 1985) and (2) the trend of rodent ambulation as a function of the test time may vary according to the nature of the measuring apparatus used. It is not clear from the reports of these authors whether this function was evaluated in the bare undifferentiated cage to investigate possible differences in locomotor behaviour between drug and vehicle treated mice, after time intervals other than that used as the test time for the light/dark box exploratory apparatus.

The diazepam (0.5 mg/kg) induced significant ($P < 0.002$) increase in dark chamber activity observed in the present study (Fig. 4.1(c)) is inconsistent with the interpretation that increased activity in the apparatus represents an increased exploratory tendency produced by a shift in a light/dark conflict in favour of reduced light aversion. Other authors using a variation of this exploratory apparatus find that anxiolytic agents produce an increase in activity in the light chamber with a concomitant decrease in activity in the dark chamber (Barry et al., 1987). There is an indication of such an effect of diazepam in the present study at the highest dose tested (Figs. 4.1(b) and 4.1(c)).

The other observations made in this study do not support the interpretation that a diazepam induced increase in the number of light/dark transitions or activity in the two chambers, reflect reduced light aversion. Perhaps most importantly, the latencies to emerge from the dark to light chamber did not show a diazepam induced decrease but rather demonstrated a tendency to increase in response to the drug (Fig. 4.1(d)). The latency to emerge from a dark to light chamber has previously been employed as an index of timidity in the rat and this parameter was reduced on treatment with diazepam (Hughes, 1981).

The time spent in the light chamber (Fig. 4.1(e)), the number of rearings made in the light chamber (Fig. 4.1(f)) and the time spent in the dark chamber (Fig. 4.1(g)) did not change in response to diazepam. The lack of effect of diazepam on the time in the light chamber is in agreement with the finding of Crawley & Goodwin (1980) who report that benzodiazepine tranquillisers did not change the dark preference of the animals. In another study which employed a similar apparatus, the benzodiazepine clorazepate has been reported to increase both the time spent in the light chamber and the number of light/dark transitions made by male mice (Belzung et al., 1987). However, a clorazepate induced significant increase in transitions was not accompanied by a significant increase in the time spent in the light chamber at each of the doses tested.

In agreement with Crawley (1981), a positive correlation (r_s=0.61, Spearman-Rank, $P < 0.01$) was found between the number of rearings in the light chamber (Fig. 4.1(f)) and the number of light/dark transitions (Fig. 4.1(a)) made by vehicle treated mice but however, in this study no such correlation was observed after diazepam treatment (e.g. at 0.5 mg/kg diazepam, r_s=0.134).

Ahtee & Shillito (1970) have demonstrated a diazepam impairment of exploration in mice at drug doses which enhanced locomotor activity. Therefore, in the present study, the diazepam induced increase in light/dark transitions could be due to a secondary effect of the drug on locomotor stimulation since the three parameters, latency to emerge, time and rearings in the light chamber did not show the expected shift in response to diazepam in favour of reduced light avoidance. To examine the pharmacological specificity of the observed increase in light/dark transitions, animals treated with the psychomotor stimulant d-amphetamine were tested.

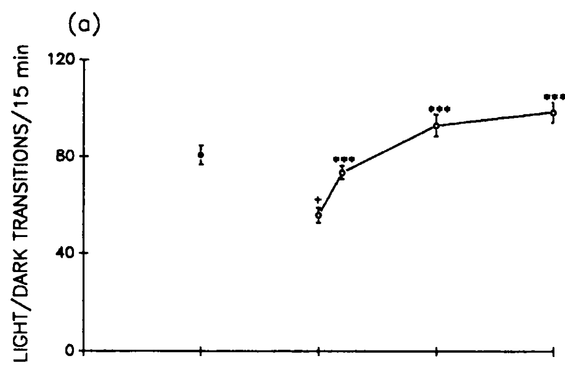
Fig. 4.2 demonstrates the effects of d-amphetamine (1 mg/kg, i.p.; 40 min) on seven parameters of the behaviour of male mice tested in the light/dark box. Injection of this drug caused a significant ($P < 0.05$) increase in the number of light/dark transitions. Injection of the drug vehicle alone had no effect on this parameter (Fig. 4.2(a)). This result is in contrast to previous

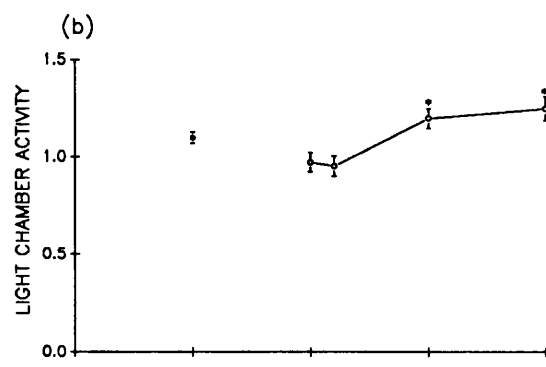
observations (see Chapter 3, Section 3.2.a) made 60 min after intraperitoneal injections of drug vehicles. Therefore, an overt response to an intraperitoneal injection is evident after 60 minutes but not after 40 minutes. As suggested elsewhere (Chapter 3, Section 3.2.b) the mechanism responsible for this effect may involve ACTH and CRF since both are known to be produced in response to mildly stressful stimuli (Haas & George, 1988) and both have apparent anxiogenic actions in the rat (File & Vellucci, 1978; Dunn & File, 1987).

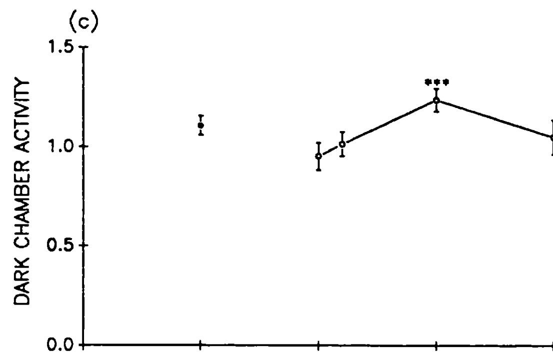
d-Amphetamine produced a significant increase in light chamber activity (Fig. 4.2(b)) and dark chamber activity (Fig. 4.2(c)). There was a tendency for the latency to emerge from dark to light chamber (Fig. 4.2(d)) and the time in the dark chamber (Fig. 4.2(g)) to increase while the time in the light chamber (Fig. 4.2(e)) and the number of rears in the light chamber (Fig. 4.2(f)) showed a tendency to decrease in response to this drug. These latter effects indicate a tendency for d-amphetamine to increase light aversion. This is consistent with other findings which report an anxiogenic action of d-amphetamine and a disruption of exploratory behaviour in rats and mice (File & Wardill, 1975; Lister, 1987b; Pellow et al., 1985; Shillito, 1970; Robbins & Iversen, 1973).

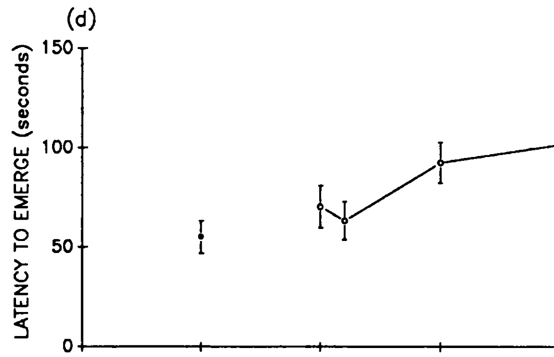
Since d-amphetamine has no anxiolytic properties but produced a change in the number of light/dark transitions similar to that observed with diazepam it is clear that an increase in this parameter does not necessarily signify an anti-anxiety drug action. Further attempts were made to investigate the extent to which this test detects an action of diazepam on exploratory behaviour as opposed to non-specific locomotor behaviour.

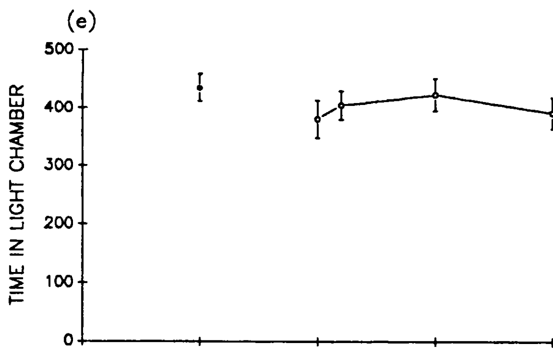
Fig. 4.1 Effects of diazepam (0.1-1 mg/kg, i.p.; 60 min) on seven parameters of the behaviour of male mice tested in the light dark box: (a) number of light/dark transitions, (b) activity in the light chamber, (c) activity in the dark chamber, (d) latency to emerge from the dark chamber, (e) time spent in the light chamber, (f) number of rearings made in the light chamber and (g) time spent in the dark chamber. Points are mean ± S.E.M. (n≥20). *$P<0.05$, ***$P<0.002$ v vehicle alone, +$P<0.05$ v untreated; Mann-Whitney U test.

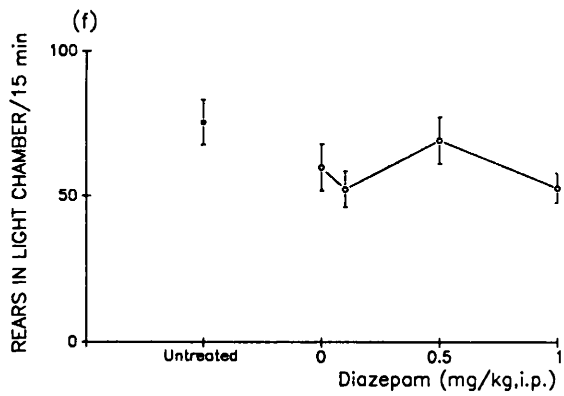

(a)
LIGHT/DARK TRANSITIONS/15 min
120
80
40
0


(b)
LIGHT CHAMBER ACTIVITY
1.5
1.0
0.5
0.0


(c)
DARK CHAMBER ACTIVITY
1.5
1.0
0.5
0.0


(d)
LATENCY TO EMERGE (seconds)
150
100
50
0


(e)
TIME IN LIGHT CHAMBER
500
400
300
200
100
0


(f)
REARS IN LIGHT CHAMBER/15 min
100
50
0
Untreated
0
0.5
1
Diazepam (mg/kg,i.p.)

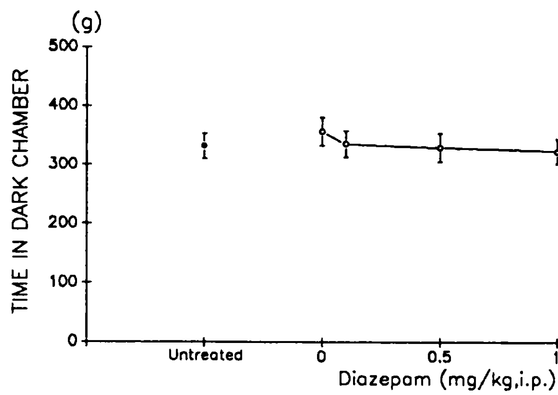
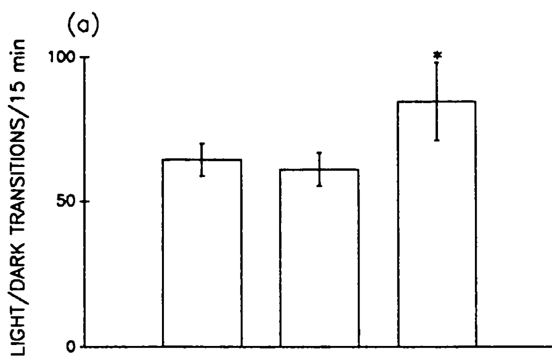
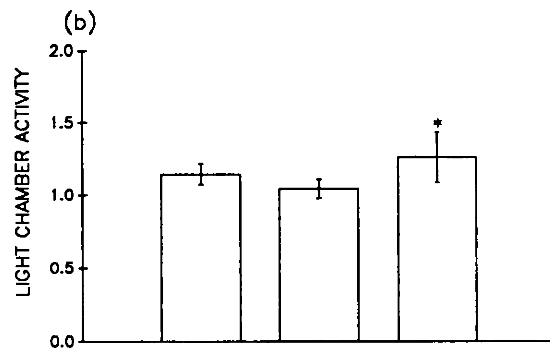
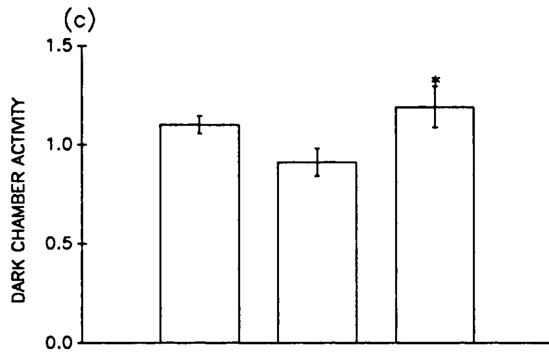
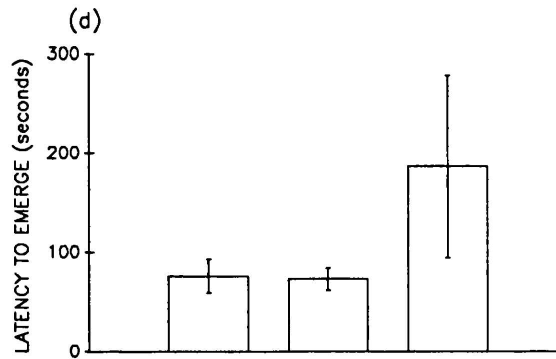

(g)
TIME IN DARK CHAMBER
500
400
300
200
100
0
Untreated
0
0.5
1
Diazepam (mg/kg,i.p.)

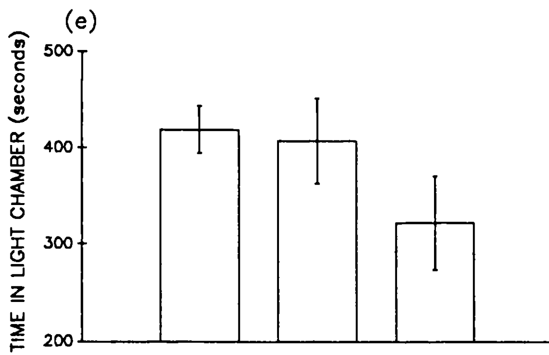
Fig. 4.2 Effects of d-amphetamine (1 mg/kg, i.p.; 40 min) on seven parameters of the behaviour of male mice tested in the light/dark box: (a) number of light/dark transitions, (b) activity in the light chamber, (c) activity in the dark chamber, (d) latency to emerge from the dark chamber, (e) time spent in the light chamber, (f) number of rearings made in the light chamber, (g) time spent in the dark chamber. Points are mean ± S.E.M. ($n \geq 7$).*$P<0.05$ **v** vehicle alone; Mann-Whitney U test.

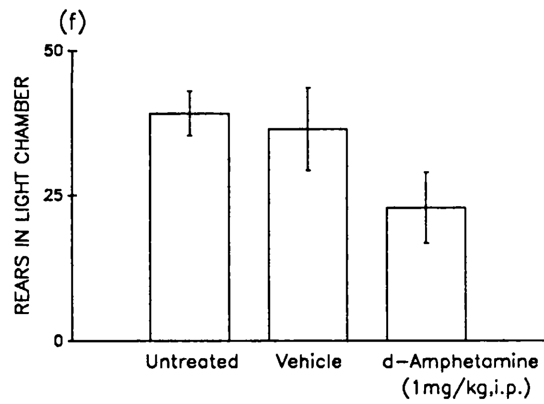

(a)
LIGHT/DARK TRANSITIONS/15 min
100
50
0
*

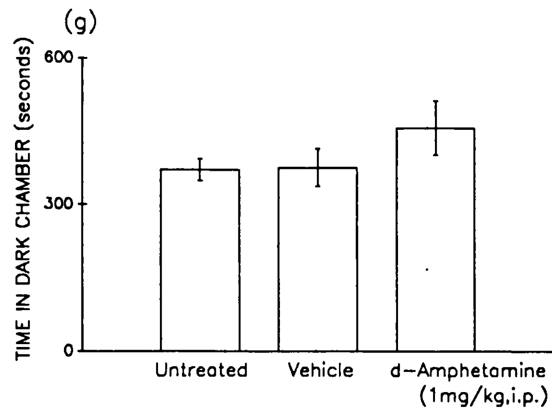

(b)
LIGHT CHAMBER ACTIVITY
2.0
1.5
1.0
0.5
0.0
*


(c)
DARK CHAMBER ACTIVITY
1.5
1.0
0.5
0.0
*


(d)
LATENCY TO EMERGE (seconds)
300
200
100
0


(e)
TIME IN LIGHT CHAMBER (seconds)
500
400
300
200


(f)
REARS IN LIGHT CHAMBER
50
25
0
Untreated
Vehicle
d-Amphetamine
(1mg/kg,i.p.)


(g)
TIME IN DARK CHAMBER (seconds)
600
300
0
Untreated
Vehicle
d-Amphetamine
(1mg/kg,i.p.)

4.2.b Attempts to distinguish effects of diazepam on exploratory behaviour from non-specific locomotor behaviour in the light/dark box apparatus

Gray (1978), using a similar apparatus to investigate the behaviour of oestrus mice, succeeded in distinguishing exploratory behaviour from non-specific locomotion by comparing the latencies of mice to enter the light chamber from the dark chamber with the latencies to enter the dark chamber from the light chamber. The hypothesis proposed by this author is that an increase in locomotory behaviour will be accompanied by a decrease in the latency to emerge from dark to light chamber with a parallel decrease in the latency to emerge from light to dark chamber. However, an increase in exploratory behaviour will be accompanied by a decrease in the latency to emerge from dark to light chamber with a parallel increase in the latency to emerge from light to dark chamber.

Fig. 4.3 shows the effects of diazepam (0.1-1.0 mg/kg, i.p.; 60 min) on the seven test parameters when tests were alternately started by placing the mouse in the dark chamber or in the light chamber. There was no significant difference between latencies to emerge from dark to light chamber and latencies to emerge from light to dark chamber, although the former tended to be higher. As shown previously, this parameter did not show any significant change in response to diazepam as compared to vehicle control (Fig. 4.3(d)).

The number of light/dark transitions increased in a dose -dependent fashion when tests were started in the light and in the dark chamber (Fig. 4.3(a)). When tests were started in the light chamber, activity in this chamber increased significantly in response to 0.5 ($P < 0.01$) and 1.0 ($P < 0.05$) mg/kg diazepam. For tests started in the dark chamber this parameter did not change significantly in response to diazepam (Fig. 4.3(b)) which is in contrast to the results described above (Section 4.2.a, Fig. 4.1(b)). Dark chamber activity showed a diazepam induced significant ($P < 0.01$) increase at 0.5 mg/kg, only when tests were started in the dark chamber (Fig. 4.3(c)).

The other three test parameters, time in the light chamber (Fig. 4.3(e)), rears in the light chamber (Fig. 4.3(f)) and time in the dark chamber (Fig. 4.3(g)) were not affected by diazepam when tests were started in the light chamber or in the dark chamber.

Mice treated with the drug vehicle alone or 0.1 mg/kg diazepam spent significantly ($P < 0.05$) more time in the light chamber when tests were started in the light chamber than when tests were started in the dark chamber (Fig. 4.3(e)). At the 0.1 mg/kg dose of diazepam, mice made significantly ($P < 0.05$) more rears in the light chamber when tests were started in this chamber compared to tests started in the dark chamber (Fig. 4.3(f)). Mice treated with the drug vehicle alone or with 0.1 or 1.0 mg/kg diazepam spent significantly ($P < 0.05$) more time in the dark chamber when tests were started in the dark chamber compared to tests started in the light chamber (Fig. 4.3(g)). These findings indicate a preference of treated mice for the chamber in which they were first placed but however diazepam does not influence this behaviour compared to vehicle control.

The effects of diazepam on the latency to emerge and four other behavioural measures were independent of start chamber. However, since this and previous experiments were conducted during normal daylight it could not be concluded that the test was not detecting an anti-anxiety action of diazepam for two reasons: (1) In order to measure a true exploratory action it could be necessary to conduct the tests during the animals active phase. (2) The light/dark contrast of the box may have been insufficient against the background illumination to reveal any significant drug induced change in light aversion.

The above possibilities were investigated by applying Gray's hypothesis during reversed daylight conditions. Fig. 4.4 demonstrates the effects of diazepam on six of the behavioural parameters when tests were alternately started by placing the mouse in the dark or light chamber during reversed daylight. From Fig. 4.4(d) it is clear that dark to light latencies were significantly ($P < 0.05$) longer than light to dark latencies. At 0.1 mg/kg,

diazepam significantly ($P < 0.05$) increased the dark to light latency compared to vehicle control. When tests were started in the dark chamber this dose of diazepam produced a decrease in the time spent in the light chamber (Fig. 4.4 (e)). These results suggest a photophobic effect of diazepam in the presence of an enhanced light/dark contrast. A similar effect of diazepam under normal daylight conditions was observed in the study of female mice at metoestrus I, described in the previous chapter, where a low dose of diazepam significantly increased the latency to emerge from the dark chamber and significantly decreased the time and number of rearings made in the light chamber (Chapter 3, Section 3.2.c, Fig. 3.4). Also, Cooper (1983, 1985b) has reported two anomalous effects of low doses of diazepam in reducing liquid consumption and ambulation in the rat.

The time in the light chamber did not change in response to diazepam when tests were started in the light chamber. Mice treated with 0.1 mg/kg diazepam spent significantly ($P < 0.05$) more time in the light chamber when tests were started in the light chamber compared to tests started in the dark chamber (Fig. 4.4(e)). Diazepam did not affect the time in the dark chamber when tests were started in the dark chamber or in the light chamber. At 0.1 mg/kg diazepam, mice spent significantly ($P < 0.05$) more time in the dark chamber when tests were started in the dark chamber compared to tests started in the light chamber (Fig. 4.4(f)).

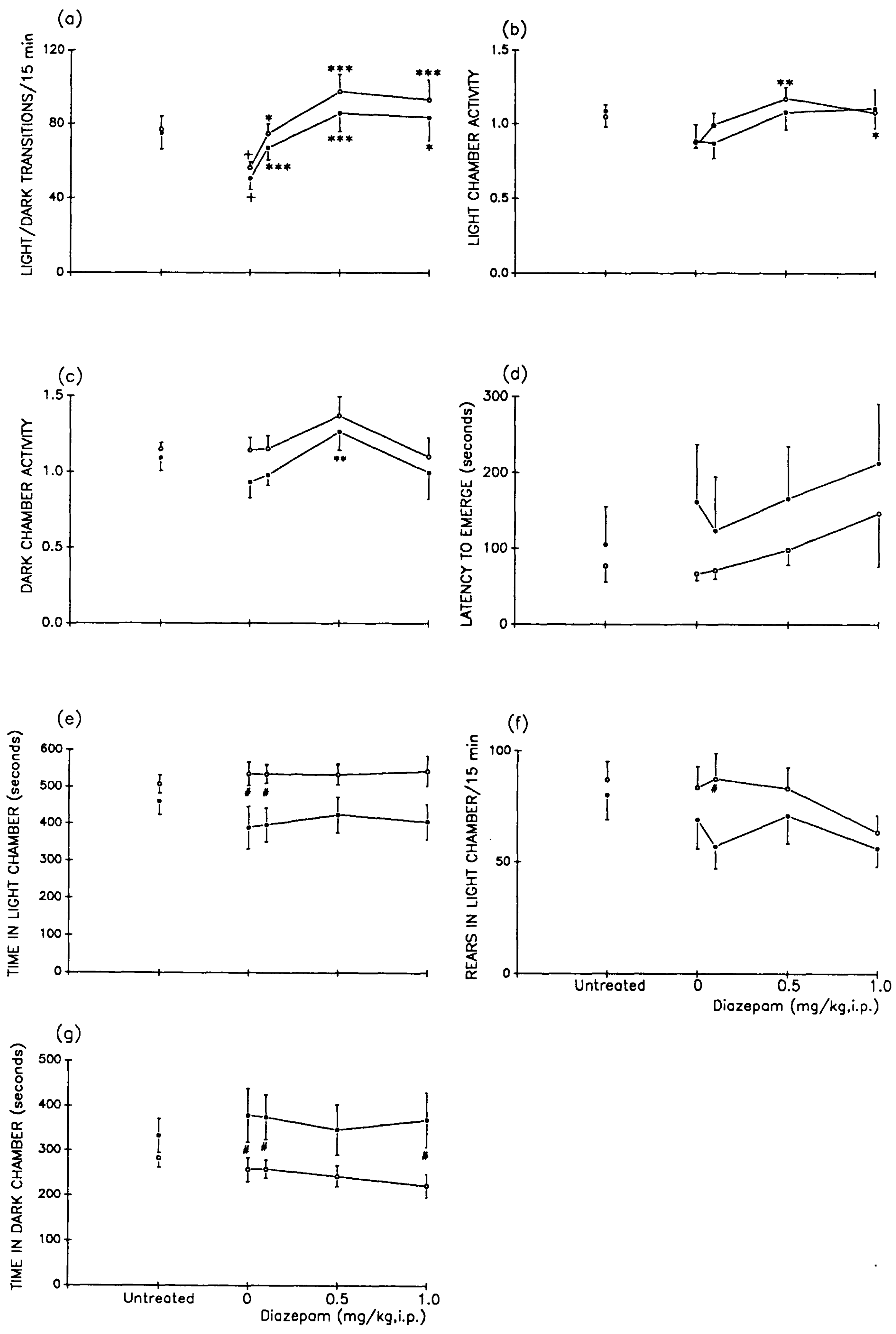
The diazepam induced increase in light/dark transitions did not reach significance for tests started in the dark chamber. For tests started in the light chamber the increase in this parameter in response to diazepam, only reached significance ($P < 0.05$) at 1.0 mg/kg. The number of transitions made by untreated mice was significantly ($P < 0.001$) higher when tests were started in the light chamber compared to tests started in the dark chamber (Fig. 4.4(a)). This may reflect the aversive nature of the light start chamber and resultant escape tendencies. The baseline number of transitions as measured in vehicle treated animals was higher than that observed during normal daylight testing (Fig. 4.3(a)) as might be expected from the normal diurnal patterns of activity of these animals. This ceiling effect would account for the failure to observe a

significant diazepam dose response whilst employing this test condition. Blumstein & Crawley (1983) using the same C57BL strain of mice report no circadian variability in this parameter in response to benzodiazepines. In contrast to tests conducted during normal daylight, an effect of the drug vehicle alone on the number of light/dark transitions was not evident (Fig. 4.4(a)). This result may reflect a change in the animal's susceptibility to a stressful stimulus in response to circadian fluctuations in CRF which are known to occur in the mouse and rat (Ungar, 1964; Hiroshige et al., 1973).

Dark chamber activity did not change in response to diazepam when tests were started in the light or dark chambers. Mice treated with 0.1 mg/kg diazepam showed significantly ($P < 0.05$) more activity in this chamber when tests were started in the light chamber compared to tests started in the dark chamber (Fig. 4.4(c)). Diazepam at a dose of 0.5 mg/kg produced a significant ($P < 0.05$) increase in light chamber activity when tests were started in the light chamber. Mice from this group also showed significantly ($P < 0.05$) higher activity compared to those similarly treated but tested by starting in the dark chamber (Fig. 4.4(b)). From these results and those obtained under normal daylight conditions it is evident that light and dark chamber activities do not show consistent changes with diazepam treatment.

The application of Gray's hypothesis did not succeed in distinguishing a diazepam induced locomotory effect from an effect on exploration. However, the experiments conducted during reversed daylight indicated that this drug produced an increase in light avoidance rather than a decrease. In view of this finding an investigation of the association between a change in test parameters and a light/dark conflict was conducted. The experiment designed to examine this association is described in the next section.

Fig 4.3 Effects of diazepam (0.1-1.0 mg/kg, i.p.; 60 min) on seven parameters of behaviour of male mice in the light/dark box when tests were started by placing the mouse in the dark chamber (closed symbol) or in the light chamber (open symbol): (a) number of transitions, (b) light chamber activity, (c) dark chamber activity, (d) latency to emerge from dark chamber,(e) time in the light chamber, (f) number of rearings in the light chamber and (g) time in the dark chamber. Points are mean ± S.E.M. (n>11). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.002$ **v** vehicle, $+P<0.05$ **v** untreated, #$P<0.05$ start in light **v** start in dark; Mann-Whitney U test.


(a)
LIGHT/DARK TRANSITIONS/15 min
(b)
LIGHT CHAMBER ACTIVITY
(c)
DARK CHAMBER ACTIVITY
(d)
LATENCY TO EMERGE (seconds)
(e)
TIME IN LIGHT CHAMBER (seconds)
(f)
REARS IN LIGHT CHAMBER/15 min
Untreated
Diazepam (mg/kg,i.p.)
(g)
TIME IN DARK CHAMBER (seconds)
Untreated
Diazepam (mg/kg,i.p.)

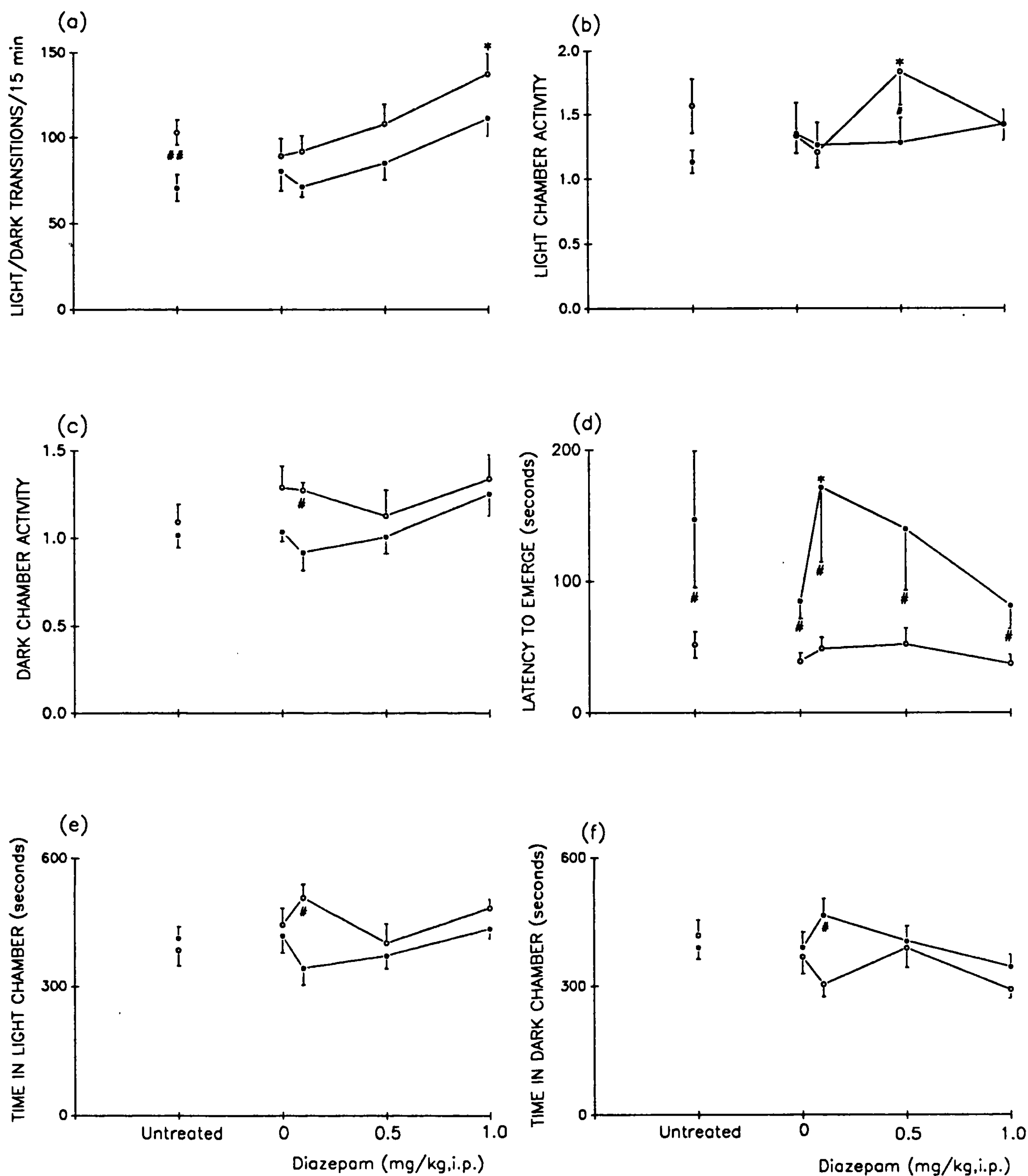


Fig. 4.4 Effects of diazepam (0.1-1.0 mg/kg,i.p.; 60 min) on six parameters of behaviour of male mice in the light/dark box when tests were started by placing the mouse in the dark chamber (closed symbol) or in the light chamber (open symbol), under reversed daylight conditions: (a) number of light/dark transitions, (b) activity in the light chamber, (c) activity in the dark chamber, (d) latency to emerge from the dark chamber, (e) time spent in the light chamber, (f) time spent in the dark chamber. Points are mean ± S.E.M. ($n \geq 9$). *$P<0.05$ **v** vehicle alone, #$P<0.05$, ##$P<0.001$ start in light **v** start in dark; Mann-Whitney U test.

4.2.c The contribution of a light/dark conflict to the changes detected in response to diazepam in the light/dark box apparatus

The role of the light/dark conflict in producing diazepam induced changes in test parameters was investigated by comparing results from tests conducted alternately in the presence or absence of a light/dark contrast. To avoid the ceiling effect in light/dark transitions observed during reversed daylight testing this experiment was carried out during normal daylight. In the absence of a light/dark contrast the apparatus was evenly illuminated by two fluorescent lamps (2 x 15 W). Tests were initiated by placing the mouse in the dark chamber (light/dark contrast) or the smaller of the illuminated chambers (even illumination). The results of this experiment are shown in Fig. 4.5.

Consistent with earlier results, in the presence of a light/dark contrast, diazepam (0.1-1.0 mg/kg) significantly ($P < 0.05$) increased the number of transitions (Fig. 4.5(a)). However, in the absence of contrast diazepam also increased this parameter with the increase becoming significant ($P < 0.05$) at 0.5 and 1.0 mg/kg. Mice treated with 0.5 mg/kg diazepam showed significantly ($P < 0.05$) more transitions in the presence of contrast compared to those tested under conditions of even illumination. An effect of the drug vehicle alone on the number of transitions was evident under both test conditions (Fig. 4.5(a)).

In the absence of contrast, diazepam (0.1-1.0 mg/kg) significantly ($P < 0.05$) increased the latency to emerge from start chamber. As found previously, the diazepam induced increase in dark to light latency was not significant (Fig. 4.5(d)). The time in the large chamber did not change in response to diazepam when contrast was present or under conditions of even illumination (Fig. 4.5(e)). In the presence of a light/dark contrast diazepam at 0.5 mg/kg increased the number of rears made in the light chamber but the increase was not significant. When the box was evenly illuminated diazepam had no effect on the number of rearings made in the large chamber (Fig. 4.5(f)). The time in the start chamber (Fig. 4.5(g)), large chamber activity (Fig. 4.5(b)) and start

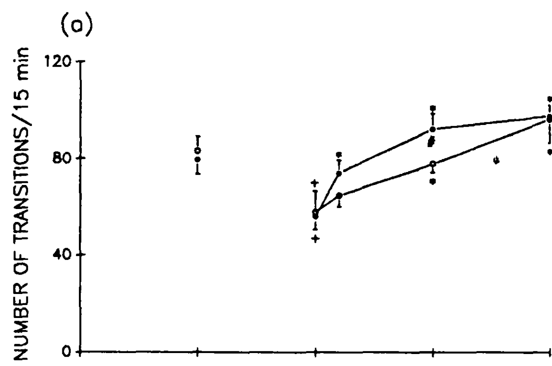
chamber activity (Fig. 4.5(c)) did not change in response to diazepam under either test condition.

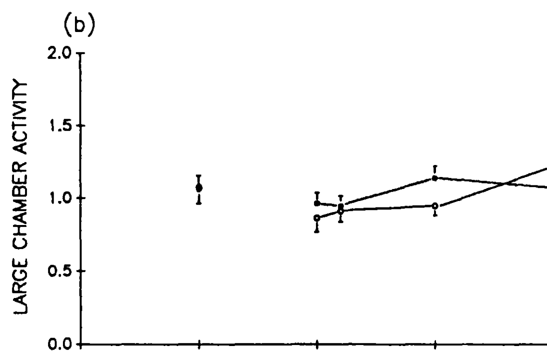
Since a diazepam induced increase in the number of chamber transitions could still be observed under conditions of even illumination (Fig. 4.5(a)) this provides further support that this is a locomotory effect rather than an anti-anxiety effect of diazepam, as reflected by a decrease in a light/dark conflict. However, the failure to detect a significant effect of 0.1 mg/kg diazepam on this parameter in the absence of contrast, together with the finding that at 0.5 mg/kg diazepam, the increase in transitions is significantly ($P < 0.05$) greater under conditions of contrast than even illumination, suggest that an increase in this parameter in response to the intermediate doses of diazepam tested, might reflect some anti-conflict drug action. The anti-conflict component could be obscured at higher drug doses as non-specific locomotory effects are enhanced. Such non-specific stimulatory effects might also explain the observed drug induced increase in the latencies to emerge from start chamber. A stimulatory effect of diazepam on these animals could result in non-specific ambulation around the start chamber until such time as the tunnel leading to the large chamber, is discovered. Alternatively, the diazepam induced increase in the latency to emerge might be interpreted as a drug induced exploration of the start chamber. However, a marked effect of diazepam on this parameter has not been observed consistently in the present study. Under normal daylight test conditions, this action of the drug assumes significance only when the apparatus is evenly illuminated (Fig 4.5(d)). This observation cannot be explained by the light induced enhanced complexity of the start chamber since a similar result was not obtained when tests were started by placing the mouse in the light (large) chamber (Section 4.2.b, Fig. 4.3(d)).

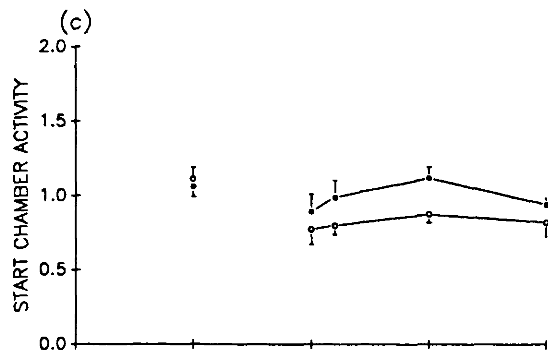
In conclusion the results from the above experiment show that the proposed light/dark conflict of this test bears little reflection on the effects of diazepam on the behavioural parameters measured. Since the behaviour of untreated mice showed no difference in the presence or absence of a light/dark contrast the possibility existed that the test does not detect changes in

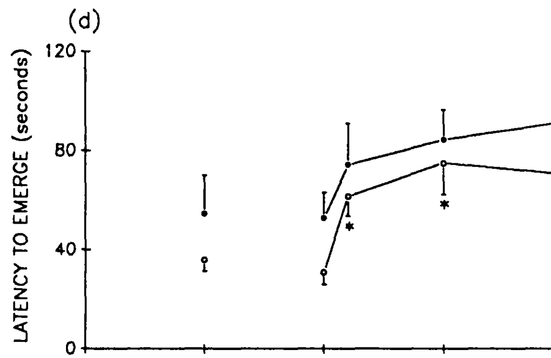
exploratory behaviour which are dependent on a light/dark conflict. This possibility was investigated and the results of this study are described in the following section.

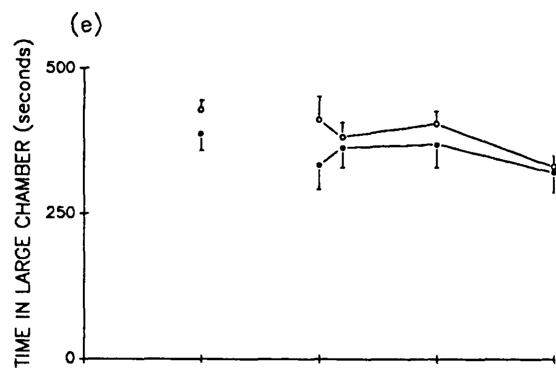
Fig. 4.5 Effects of diazepam (0.1-1.0 mg/kg, i.p.; 60 min) on seven parameters of behaviour of male mice in the exploratory apparatus in the presence of a light/dark contrast (closed symbols) or even bright illumination (open symbols): (a) number of transitions, (b) activity in the large chamber, (c) activity in the smaller chamber, (d) latency to emerge from the smaller chamber, (e) time spent in the larger chamber, (f) number of rearings made in the larger chamber and (g) time spent in the smaller chamber. Points are mean ± S.E.M. ($n \geq 10$).*$P<0.05$ **v** vehicle, +$P<0.05$ **v** untreated, #$P<0.05$ light/dark contrast **v** even illumination; Mann-Whitney U test.

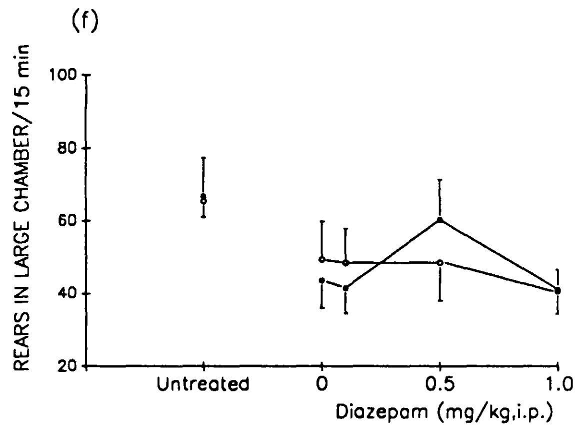

(a)
NUMBER OF TRANSITIONS/15 min
120
80
40
0

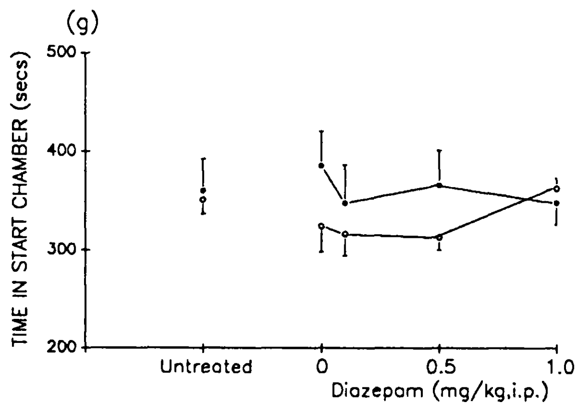

(b)
LARGE CHAMBER ACTIVITY
2.0
1.5
1.0
0.5
0.0


(c)
START CHAMBER ACTIVITY
2.0
1.5
1.0
0.5
0.0


(d)
LATENCY TO EMERGE (seconds)
120
80
40
0


(e)
TIME IN LARGE CHAMBER (seconds)
500
250
0


(f)
REARS IN LARGE CHAMBER/15 min
100
80
60
40
20
Untreated
0
0.5
1.0
Diazepam (mg/kg,i.p.)


(g)
TIME IN START CHAMBER (secs)
500
400
300
200
Untreated
0
0.5
1.0
Diazepam (mg/kg,i.p.)

4.2.d Habituation of male mice to the light/dark box apparatus and the effects of diazepam or d-amphetamine treatment on habituated animals.

In an attempt to elucidate the relation of parameter changes of this test to exploratory behaviour, the effects of repeated testing on these parameters and the effects of diazepam (0.5 mg/kg, i.p.; 60 min) on test habituated animals were examined. For comparison, the effect of the psychomotor stimulant d-amphetamine (1 mg/kg, i.p.; 40 min) on the number of light/dark transitions made by test habituated animals was also studied.

Animals were given a daily 15 min test session in the apparatus for 5 consecutive days. On day 6 the animals were randomly assigned to the following treatment groups, untreated, saline vehicle, propylene glycol/ethanol vehicle, d-amphetamine (1 mg/kg) or diazepam (0.5 mg/kg).

Fig. 4.6 demonstrates the effect of test familiarisation on untreated animals and the effect of diazepam or its vehicle on test experienced animals. The number of light/dark transitions decreased significantly ($P < 0.05$) from day 1 to day 3 and thereafter showed no change in untreated animals. Treatment of test familiar animals with diazepam on day 6 produced a significant ($P < 0.05$) increase in the number of transitions as compared to vehicle control (Fig 4.6(a)).

There was a progressive decrease in the dark to light latency from day 1 to day 6 in untreated animals. This decrease was significant ($P < 0.01$) from day 1 to day 3 (Fig 4.6(d)).

The time spent in the light chamber increased significantly ($P < 0.01$) from day 1 to day 2 and thereafter showed no change (Fig. 4.6(e)). The time in the dark chamber showed a non-significant decrease from day 1 to day 2 but over the 5 trials this parameter remained largely unaffected in untreated animals (Fig. 4.6(g)).

The number of rearings made in the light chamber had increased by day 3. This increase fell short of being significant ($P < 0.058$) and after day 3 this parameter remained unchanged (Fig. 4.6(f)). Both light chamber activity (Fig. 4.6 (b)) and dark chamber activity (Fig. 4.6(c)) had decreased significantly ($P < 0.01$) by day 2 and showed no further significant decrease in untreated animals.

Treatment of test habituated animals with 0.5 mg/kg diazepam did not affect any of the test parameters (Fig. 4.6(b-g)) with the exception of light/dark transitions (Fig. 4.6(a)).

Fig.4.7 shows the effect of 1 mg/kg d-amphetamine on the number of light/dark transitions made by mice habituated to the test for 5 days. As found before, in untreated animals this parameter decreased significantly ($P < 0.05$) from day 1 to day 2. Treatment with d-amphetamine on day 6 produced a decrease which was not significantly different from vehicle control. This drug did not affect the other test parameters in test habituated animals.

The above results show that the behavioural parameters of time in the light chamber (Fig. 4.6(e)), dark to light latency (Fig. 4.6(d)) and rears in the light chamber (Fig. 4.6(f)) can change progressively on test habituation in a manner which would suggest decreased light aversion. However, these indices of increased exploration of the light chamber and decreased aversion to the test situation are accompanied by a decrease in both the number of light/dark transitions (Fig. 4.6(a)) and light and dark chamber activity (Fig. 4.6(b-c)). This finding indicates that exploratory behaviour occurs when general locomotor activity in the apparatus is reduced. The observed change in locomotion is consistent with the decrement in locomotor behaviour known to occur in the open field situation on repeated trials carried out on consecutive days (see Archer, 1973).

The direction of change in test parameters observed in untreated animals on habituation is in marked contrast with that previously found from

tests of diazepam treated naive animals. While untreated animals familiarised to the test showed a decrease in the number of light/dark transitions and a shift in other parameters indicative of reduced light aversion, diazepam treatment of naive animals produced an increase in transitions and no change in the other parameters which would suggest an increased exploration of the light chamber. This finding together with the diazepam induced increase in light/dark transitions observed in test habituated animals strongly suggests that this parameter change in response to diazepam is due largely to locomotor stimulation. However, this interpretation must be reconciled with the finding that treatment of test habituated animals with the stimulant d-amphetamine does not produce an increase in transitions, as seen in naive animals (Section 4.2.a, Fig 4.2(a)), but rather induces a decrease in this parameter.

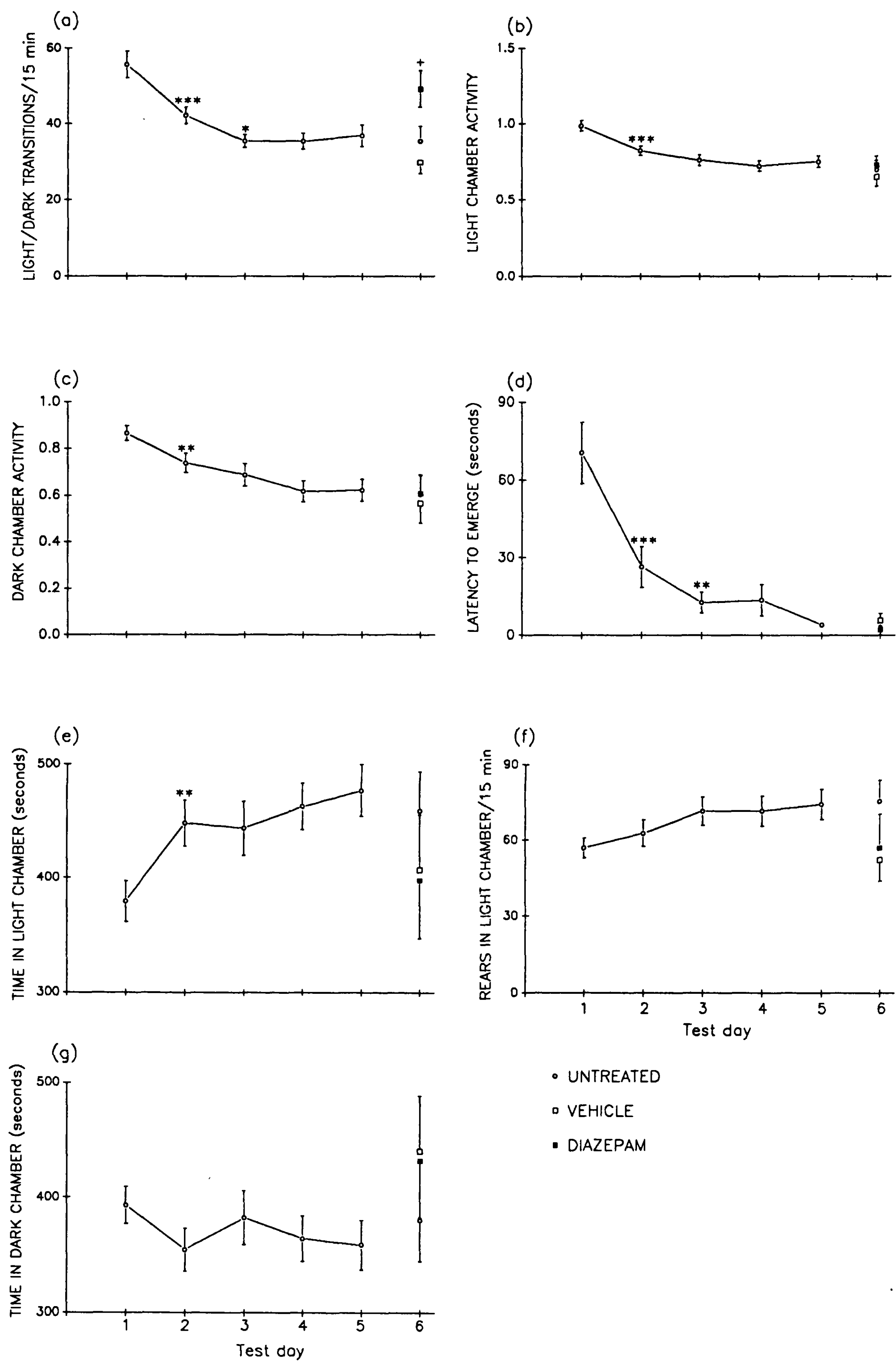
The possibility that the diazepam induced increase in transitions in test familiar animals reflects a true exploratory action which is independent of change in other test parameters is unlikely in view of the progressive changes seen in these parameters on habituation; changes which would suggest a decrease in light aversion and an increased exploration of the light chamber. The possibility that the diazepam induced increase in transitions seen in habituated animals is due to an amnesic effect of the drug is also unlikely since the latency parameter was not affected in these animals. The low level of this parameter attained in habituated animals would suggest a high degree of familiarity and learning and would rule against a drug induced disruption of this parameter through either locomotor stimulation or amnesia. Such high levels of familiarity would also preclude any further increase in exploration, providing additional support for the suggestion that the diazepam induced change in transitions is largely the result of locomotor stimulation.

Other workers have shown that administration of chlordiazepoxide to test experienced rats produced a significant increase in open field ambulation but not in exploratory activity of a Y maze (Christmas & Maxwell, 1970; Marriott & Spencer, 1965). In the present study, the discrepancy found between the effects of diazepam and d-amphetamine on test familiar animals,

most likely reflects a difference in the interaction between test experience and drug action. The result obtained with d-amphetamine is in agreement with that of Steinberg et al. (1961) who demonstrated that amphetamine decreased exploration of a Y maze in test experienced rats; an effect opposite to that found with test naive rats.

In conclusion the data obtained from untreated habituated animals indicate that this test is sensitive to a shift in a light/dark conflict in favour of reduced light aversion. This sensitivity becomes apparent when the background level of locomotion is reduced. The inability of the test to detect such an anti-conflict action of diazepam in test naive animals could be due to interference by other effects of this drug. The results from the present evaluation suggest that two of these factors appear to be a non-specific stimulant effect and photophobia.

Fig. 4.6 Effects of repeated testing on the behaviour of untreated mice in the light/dark box and the effects of diazepam (0.5 mg/kg, i.p.; 60 min) on test experienced animals: (a) number of light/dark transitions, (b) activity in the light chamber, (c) activity in the dark chamber, (d) latency to emerge from dark chamber, (e) time spent in the light chamber, (f) number of rearings made in the light chamber and (g) time spent in the dark chamber. Points are mean ± S.E.M. (n=44; and ≥14 for each treatment). *P<0.05, **P<0.01, ***P<0.002 **v** previous test day, +P<0.05 **v** vehicle alone; Mann-Whitney U test.


(a)
LIGHT/DARK TRANSITIONS/15 min
60
40
20
0
***
*
(b)
LIGHT CHAMBER ACTIVITY
1.5
1.0
0.5
0.0
***
(c)
DARK CHAMBER ACTIVITY
1.0
0.8
0.6
0.4
0.2
0.0
**
(d)
LATENCY TO EMERGE (seconds)
90
60
30
0
***
**
(e)
TIME IN LIGHT CHAMBER (seconds)
500
400
300
**
(f)
REARS IN LIGHT CHAMBER/15 min
90
60
30
0
1
2
3
4
5
6
Test day
(g)
TIME IN DARK CHAMBER (seconds)
500
400
300
1
2
3
4
5
6
Test day
UNTREATED
VEHICLE
DIAZEPAM

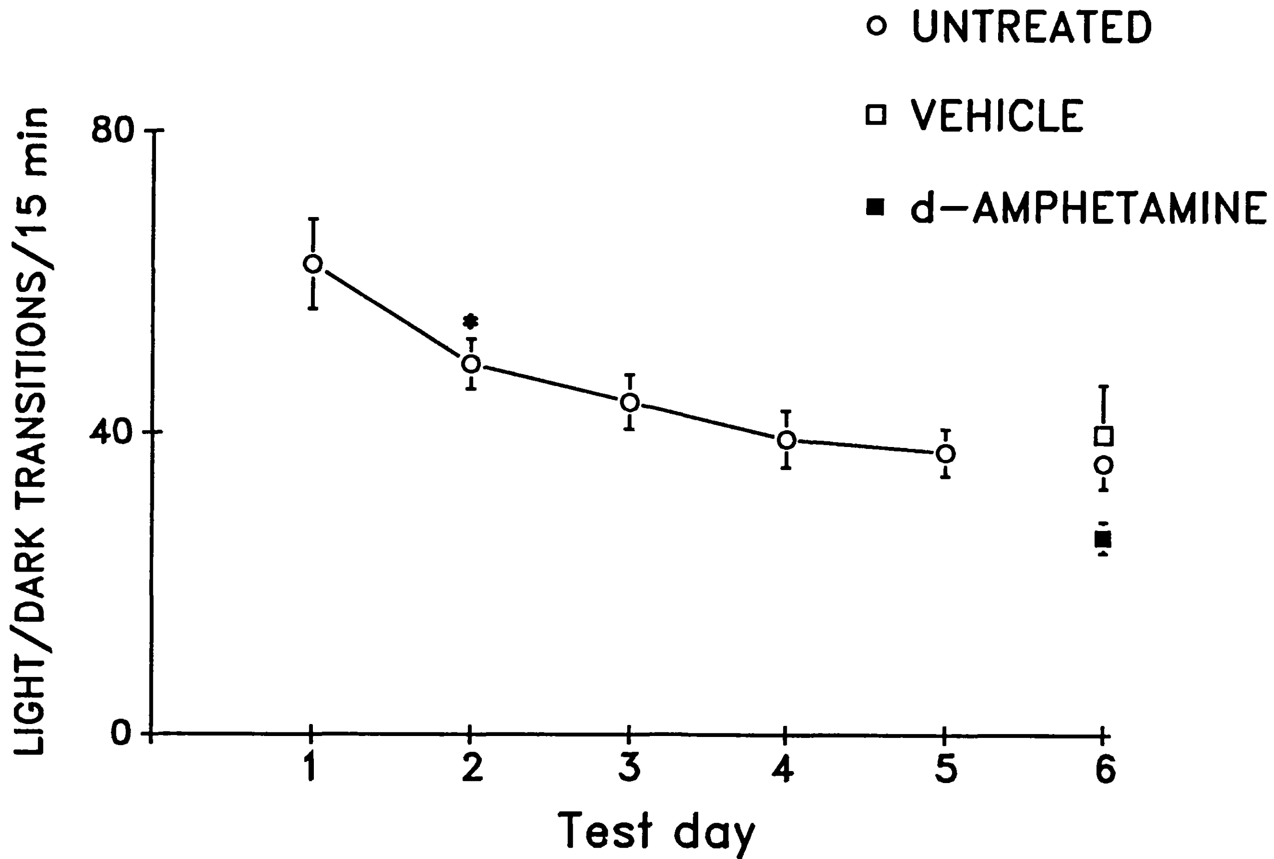


Fig. 4.7 Effect of repeated testing in the light/dark box on the number of light/dark transitions made by untreated mice and the effect of d-amphetamine on this parameter in test experienced animals. Points are mean ± S.E.M. (n=18; and =6 for each treatment). *P<0.05 **v** test day 1; Mann-Whitney U test.

4.3 Conclusions

In agreement with previous reports (Crawley, 1981) diazepam (0.1-1.0 mg/kg) reliably produced an increase in the number of light/dark transitions made by male mice in the light/dark box. However, the drug induced increase in transitions was not accompanied by changes in other test parameters indicative of reduced light aversion and increased exploration of the brightly lit chamber. An increase in the number of light/dark transitions is not specific to an anxiolytic action of diazepam since the same result was obtained after treatment of mice with the psychomotor stimulant d-amphetamine. Further analysis of this action of diazepam revealed that it is not dependent on the presence of a light/dark conflict since similar changes were observed when the apparatus was evenly illuminated. However, results from this experiment indicate that such a response to diazepam might reflect some anti-conflict action since the magnitude of the response was greater at the lower doses tested in the presence of a light/dark conflict than when the apparatus was evenly illuminated. Nevertheless, subsequent experiments performed in this study confirm that a diazepam induced increase in light/dark transitions is due largely to a stimulant effect of the drug. Diazepam treatment of test habituated animals, showing a high degree of familiarity to the apparatus, still produced an increase in the number of transitions but did not affect the other test parameters; a similar situation to that observed in test naive animals treated with this drug.

Familiarisation of untreated animals to the test apparatus produced a progressive decrease in the latency to emerge from dark to light chamber and a progressive increase in the time and rearings in the light chamber. These changes were accompanied by a decrease in both light and dark chamber activities and the number of light/dark transitions. These results demonstrate that the test is sensitive to a shift in a light/dark conflict in favour of reduced light aversion. However, exploration of the brightly lit chamber does not become apparent until the general level of locomotion is reduced. Since the test is sensitive to the stimulant action of diazepam, this precludes detection of an anti-conflict action of the drug. A further complexity of using the light/dark box

to detect an anxiolytic action of diazepam arises from the finding that a low dose of diazepam has an apparent photophobic effect detectable by the test under conditions of reversed daylight.

In conclusion this test does appear to represent a conflict between the tendency to explore the brightly lit chamber versus the tendency to retreat into the dark chamber. In untreated mice a shift in this conflict in favour of decreased light avoidance is detectable when the level of general locomotion is reduced. Therefore, this test is unsuitable for a study of anxiety which requires test naive animals or the use of treatments such as diazepam, which induce a further increase in locomotor behaviour. This finding is in disagreement with the reports of other authors who have used a variant of this apparatus to study the effects of oestrous cycle stage or diazepam on the exploratory behaviour of mice (Gray, 1978; Barry et al., 1987). The major difference between the apparatus employed by these authors and that used in the present study is the mode of separation of the two chambers. In this study, a short tunnel was used to separate the light and dark chambers whereas the apparatus used by Gray (1978) and Barry et al. (1987) has an opening in the partition between the chambers. It is possible that the tunnel component of the test elicits a high level of non-specific locomotion which interferes with exploratory behaviour.

Evidence based on the behavioural effects of d-amphetamine in the light/dark box apparatus indicate that the test may be sensitive to the detection of anxiogenic activity which can be distinguished from a non-specific change in locomotion. Crawley et al. (1984) report that this test is insensitive to the detection of anxiogenic activity of benzodiazepine inverse agonists and partial inverse agonists. However, this conclusion appears to be based exclusively on the failure to detect a change in the number of light/dark transitions. Contrary to this report, Belzung et al. (1987) have demonstrated a decrease in the time spent in the light chamber and the number of light/dark transitions made by male mice in response to the benzodiazepine inverse agonist ßCCM. Since the effect of this drug on the former parameter was significant at a dose which did not produce a significant decrease in the number of transitions, this finding corroborates the results derived from the

present evaluation which show that the number of light/dark transitions may function as an index of locomotor behaviour and thereby distinguish a change in exploration from a non-specific motor effect.

Chapter 5

The development of a pharmacological conditioning procedure in the pig sensitive to changes in psychological state

5.1 Introduction

5.2 Results and discussion

5.2.a Establishment of a conditioning procedure to produce selection of one lever only following either saline or PTZ treatment.

5.2.b Establishment of a conditioning procedure to produce selection of both levers alternately following saline treatment and selection of one lever only following PTZ treatment.

5.3 Conclusions

5.1 Introduction

The psychological state induced by centrally acting drugs can be studied using drug discrimination paradigms (see Lal, 1977). At subconvulsant doses, pentylenetetrazole (PTZ) is anxiogenic in humans (Rodin, 1958) and accordingly the discriminative stimulus induced by PTZ in rats has been proposed as an animal analogue of anxiety (Shearman & Lal, 1979). The possibility that the PTZ discriminative stimulus may correspond to the stimulant or proconvulsant properties of the drug has been investigated by examining the pharmacological specificity of this stimulus. Such investigations have revealed that a generalisation to or an antagonism of the PTZ cue is selective for drugs with anxiogenic or anxiolytic properties, respectively (Shearman & Lal, 1979, 1980; Stephens et al., 1984; Vellucci et al., 1988). For instance, the psychomotor stimulant d-amphetamine did not generalise to the PTZ cue and the GABA mimetic etomidate did not antagonise the cue, although this is an effective agent in preventing PTZ induced seizures (Shearman & Lal, 1979, 1980).

Although these pharmacological evaluations suggest that the discriminative stimulus induced by PTZ in rats relates to the anxiogenic properties of this drug, they do not provide conclusive evidence to eliminate the possibility that the cue corresponds to a proconvulsant action of PTZ. This possibility arises because anxiolytic drugs which antagonise the cue or anxiogenic drugs which mimic the cue also possess anticonvulsant or proconvulsant properties, respectively. Thus validation of the PTZ cue as anxiogenic would require corroborative evidence from a non-pharmacological investigation employing a specific anxiogenic or anxiolytic stimulus.

The aim of the present study was to determine if PTZ could induce a discriminative stimulus in pigs. An attempt was made to evaluate and improve the PTZ conditioning procedure, in order to conduct a behavioural evaluation of the PTZ stimulus to determine the anxiogenic nature of this stimulus.

5.2 Results and discussion

5.2.a Establishment of a conditioning procedure to produce selection of one lever only following either saline or PTZ treatment.

Acquisition of the discriminatory response

Of the 5 pigs trained to discriminate the effect of PTZ from saline according to procedure 2A (Chapter 2, section 2.3.d), two (pigs number 13 and 15) satisfied the discrimination criterion within the experimental period. The number of PTZ and saline sessions to satisfaction of criterion were 24 PTZ/12 saline and 34 PTZ/24 saline for pig 13 and pig 15 respectively. For pig 13 the criterion was achieved with training doses of PTZ of 5 and 6.25 mg/kg. The training dose of PTZ at which the criterion was achieved for pig 15 was 10 mg/kg. The entire sequence of saline and PTZ training sessions received by these two animals during the course of the experiment was analysed by the chi-square test to examine the possibility that the set discrimination criterion was maintained by means other than a distinction of the PTZ cue from the non-drug state. Other possibilities might include lever selection based on the last reinforced lever or a random process of lever selection. However, in view of the strict criterion and the schedule of training sessions employed, both possibilities would seem unlikely. A chi-square analysis of the first lever selected as a function of the lever reinforced in the previous training session, revealed that there was no significant deviation from the expected ratio of lever selections as predicted from the schedule of PTZ and saline treatments for animals showing a complete discriminatory response. This analysis is shown in Table 5.1.

Table 5.1 The frequency of the first lever selected from saline and PTZ training sessions as a function of the lever reinforced in the previous session, for pigs 13 and 15. The frequency expected if the animals showed a complete discriminatory response is shown in parentheses. $P > 0.05$ expected **v** observed frequencies; Chi-Square test.

Pig 13	First lever selected		
Lever last rewarded		A	B
	A	15 (14.2)	36 (42.6)
	B	43 (42.6)	48 (42.6)
Pig 15	**First lever selected**		
Lever last rewarded		A	B
	A	28 (29.4)	32 (29.4)
	B	30 (29.4)	8 (9.8)

The response rates of pigs 13 and 15 on the saline and PTZ designated lever before first reward, in the training sessions to satisfaction of criterion are shown in Table 5.2. For pig 15 the rate of response to first reward was significantly ($P < 0.02$) lower after PTZ treatment compared to saline treatment. There was no difference in the response rate of pig 13 between saline and PTZ treatments.

Of the three animals that did not satisfy the discrimination criterion, one did show signs of discriminatory behaviour but unfortunately had to be removed from the study due to illness.

Table 5.2 The rate of lever pressing before attainment of first reward by pigs 13 and 15 in the saline and PTZ training sessions to satisfaction of discrimination criterion. * $P < 0.02$ **v** response on the saline lever; Two-sample t test.

Pig 13		Pig 15	
Lever presses per second before first reinforcement (Mean ± S.E.M.)			
Saline lever	PTZ lever	Saline lever	PTZ lever
1.90 ± 0.06 (n=10)	1.92 ± 0.13 (n=10)	1.27 ± 0.08 (n=10)	0.96 ± 0.08 * (n=10)

The validity of the PTZ drug lever response as an index of psychological state.

A preliminary experiment revealed that administration of PTZ during a saline session to an animal capable of recognising the PTZ stimulus, would produce a change in response from selection of the saline lever to selection of the PTZ lever, but only if the drug was administered prior to attainment of the first reinforcement on the saline lever. An experiment was conducted, therefore, to investigate the possibility that the maintained response of trained animals on the PTZ lever during PTZ sessions results from a fixation on the reinforced lever rather than an association of lever selection with drug cue. For this experiment, delays were introduced between the injection of PTZ and the start of the test. Fig 5.1 shows the results of these tests conducted at three different time intervals after the administration of the training dose of PTZ (5 mg/kg) to pigs 13 and 15. The saline lever was selected by pig 13 in tests at the three time intervals of 590, 484 and 300 seconds following PTZ administration. In contrast, pig 15 selected the PTZ lever when tested at intervals of 532, 470 and 303 seconds after PTZ administration.

The above differences between pigs 13 and 15 in the time of action of PTZ might be accounted for by differences between these animals in the pharmacokinetics of PTZ and/or different sensitivities to the cueing properties of this drug. At this stage of the experiment, a very short lasting tremor was noted to occur in pig 15 in response to PTZ injections. Such an effect of PTZ was not observed in pig 13 at this time but had been found to occur at an earlier stage of the experiment. Since the magnitude of this PTZ effect did not increase on repeated administration of the training dose of PTZ to pig 15, it may not reflect an increased sensitivity to the convulsant property of PTZ (chemical kindling) but maybe a symptom of autonomic activation induced by this drug. At this stage of the experiment pig 13 may have adapted to any such peripheral aspects of the PTZ cue. A further speculation, then, might be that the cue induced by PTZ in pig 13 was more specific than that induced in pig 15. Such a difference in cue specificity would explain the present observations of differences in the duration of the PTZ stimulus between the two animals. There are no reports of such peripheral effects of PTZ but in humans, it is well known that somatic manifestations of anxiety occur which are sensitive to alleviation by ß-adrenoceptor antagonism (see Green & Costain, 1981). Although an attempt to demonstrate generalisation of the ß-adrenoceptor agonist isoprenaline to the PTZ cue in the rat has been unsuccessful (Vellucci et al.,1988), in accordance with the above speculations, this study could have been confounded by an altered specificity of the PTZ stimulus at the particular stage of the experiment when the test was conducted.

The above results show that the response of pig 13 on the PTZ lever for the duration of the 10 min training sessions, given at this stage of the experiment, is based on a fixation on this lever following first reward rather than an association with drug cue. A similar phenomenon of lever fixation following first reward also appears to occur in rats which are conditioned to discriminate PTZ from saline by an analogous procedure employing a FR schedule of reinforcement (see Lal & Emmett-Oglesby, 1983). This is also reflected by the finding that generalisation or antagonism properties of test drugs are determined from the discriminatory response of the animals before the attainment of first reward (Lal & Emmett-Oglesby, 1983; Stephens et al.,

1984). Colpaert (1977) concludes that while a discriminative stimulus controls the initial lever selected, subsequent response on the selected lever is determined primarily by the reinforcement contingency.

In order to overcome such interference of conditioned reinforcement schedules with the interpretation of test data, procedures have been developed whereby periods of non-reinforced responding are included during training to enable test sessions to be conducted in extinction (e.g. Vellucci et al., 1988). Another such procedure employed is similar to that used in the present experiments whereby response on a lever to the set FR results in test termination without reinforcement (e.g. Leidenheimer & Schechter, 1988).

These findings together with the above results obtained from pig 13 indicate that an investigation of the PTZ cue by the PTZ discrimination paradigm employed in the present study, is limited to the period of response before attainment of first reinforcement. While this time limitation would allow a pharmacological determination of the PTZ cue it would not permit certain behavioural evaluations of this stimulus such as an examination of the discriminatory response of the animals to the state induced by a behavioural conditioned anxiogenic stimulus. A comparison of the state induced by such a conditioned stimulus to the PTZ cue would provide a more definitive determination of the anxiogenic nature of this cue. A preliminary study showed that application of a conditioned stimulus (CS) to a pig for a short duration at the start of a test resulted in the development of long latencies to respond in all subsequent untreated sessions. A valid assessment, therefore, of a conditioned emotional state would require a period of stable response before presentation of the CS to ensure that a response during the application of the CS is specific to the CS and not to other non-specific associations with the conditioning procedure.

To enable such a behavioural evaluation of the PTZ stimulus, attempts were made to devise a conditioning procedure whereby the animal would readily associate lever selections with the presence or absence of the drug cue. To this end, a pilot experiment was performed in which the PTZ interoceptive

cue was substituted by an exteroceptive tone stimulus (1600 Hz). Pigs (n=2) were initially trained to select both levers alternately at a FR 40 (for details of training procedure, see Chapter 2, Section 2.3.d, Procedure 2B). On establishment of a stable alternation response at a FR 40 the animals were then trained to respond on one lever only at a FR 40 in the presence of the tone stimulus. When this discrimination was learned, tests were conducted whereby the tone stimulus was applied for 5 mins after the animal had been responding on both levers alternately. This produced a change in response from alternation of lever selection to selection of the tone lever only. Tests in which the tone stimulus was applied for 5 mins and then terminated, resulted in a change in response from selection of the tone lever only to selection of both levers alternately (results not shown).

From the above pilot study, this novel conditioning procedure appeared to offer a reliable means of assessing the detection of a discriminative stimulus, irrespective of when it was applied with regard to reinforcement on either lever. Experiments were then conducted using this procedure with PTZ instead of tone and the results obtained are described in the next section.

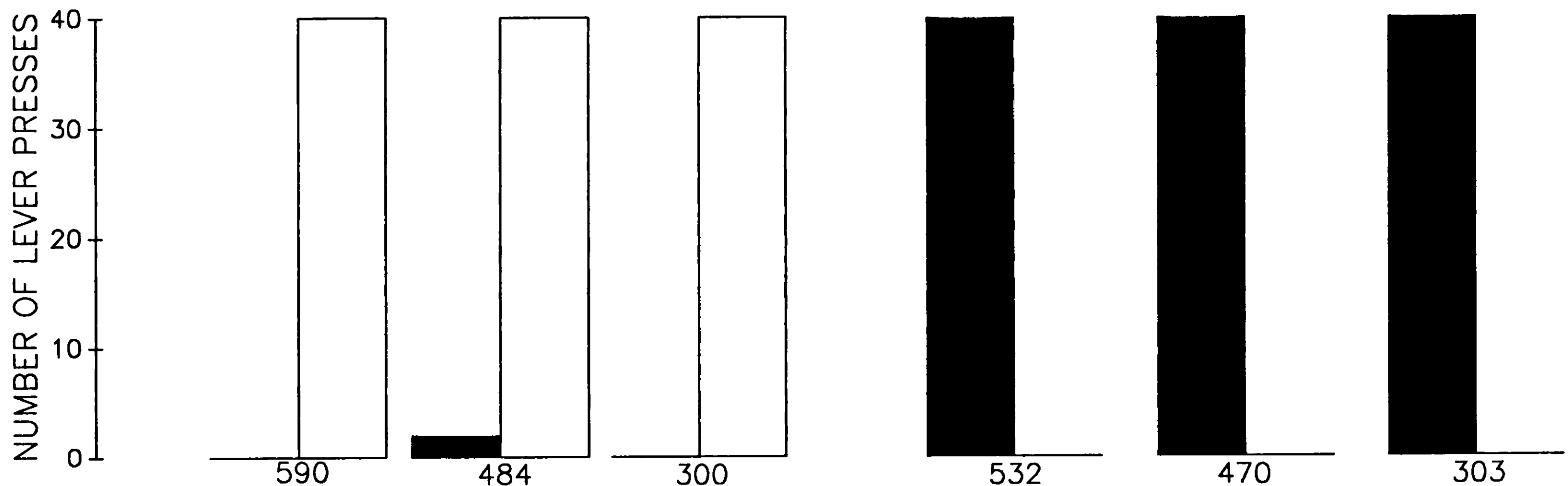


Fig. 5.1 The discriminatory response of pigs 13 and 15 at three different time intervals following injections of the training dose of PTZ (5 mg/kg, i.v.).

5.2.b Establishment of a conditioning procedure to produce selection of both levers alternately following saline treatment and selection of one lever only following PTZ treatment.

Acquisition of the discriminatory response

All four pigs pharmacologically conditioned by procedure 2B (Chapter 2, Section 2.3.d), satisfied the criterion of successful discrimination within 66 ± 14 (Mean ± S.E.M.) training sessions. Table 5.3 shows the number of saline and PTZ sessions required by each pig until a correct saline session occurred following a PTZ session. The training schedule of S P S S P P S S P S was then incorporated. The number of saline and PTZ training sessions required by each pig to satisfaction of criterion is also shown in this table. The approximate three-fold difference between the number of saline and PTZ sessions required before incorporation of the training schedule reflects the disruption of the saline alternation response until the pigs had learned to recognise the PTZ cue. The requirement for approximately twice the number of saline sessions to PTZ sessions in the satisfaction of the discrimination criterion demonstrates the greater difficulty in distinguishing the cue absent state.

Table 5.3 The number of saline and PTZ training sessions required by pigs C1, C3, C4 and C5 before the training schedule of SPSSPPSSPS was incorporated and before the criterion of successful discrimination was satisfied.

	Pig number	Number of saline sessions	Number of PTZ sessions
Number of sessions to incorporation of training schedule (SPSSPPSSPS)	C1	22	09
	C3	34	13
	C4	36	12
	C5	21	08
Number of sessions to satisfaction of discrimination criterion	C1	41	22
	C3	46	18
	C4	69	34
	C5	24	10

Table 5.4 shows the training doses of PTZ for each pig, during (a) and after (b) satisfaction of the discrimination criterion. The dose of PTZ was decreased in training when any physical symptoms such as body tremor, head twitch or extension of forelimbs, were evident to avoid the kindling effects of PTZ. If the discriminatory response became unstable at the reduced PTZ dose, the dose was subsequently increased to a dose which did not produce proconvulsant symptoms. Although, extreme caution was taken to avoid the occurrence of such symptoms, in some cases a very mild short lasting shiver like tremor of the back in response to PTZ injection was accepted if a lower dose of the drug failed to produce stable discrimination. As discussed in the previous section, such a symptom may be representative of an autonomic response to PTZ rather than an increased sensitivity to the proconvulsant or convulsant properties of the drug.

Also shown in Table 5.4 is the designated drug lever (A or B) for each pig, the mean drug lever response time and the number of training sessions associated with each training dose of PTZ. The drug lever response time represents the time from when the test was started to when the last reinforcement on the drug-designated lever was obtained. These tests were terminated either after a preset time or when the animal switched from selection of the drug lever to selection of the alternate lever. Preset test times were chosen so as to minimise the risk of training the pig on the PTZ lever in the absence of the drug cue and such times were generally determined from the animals response times on the drug lever in previous PTZ sessions.

There was a tendency for the PTZ drug lever response time to decrease as the training dose of the drug was decreased and the number of PTZ training sessions was increased. There is a two to three fold difference between PTZ response times from the earliest PTZ training sessions to the final training session of the experiment (Table 5.4b). For three of the pigs (C3, C4 and C5) the same training dose of drug (5, 2.8 and 5 mg/kg, i.v., respectively) produced shorter response times when given in a later series of training sessions. Such variations in the duration of the PTZ stimulus may be explained by differences in the pharmacokinetics of PTZ and also by a change in the cueing properties of this drug. It is possible that long term treatment with PTZ produces a change in its metabolism by the induction of liver microsomal enzymes. Such metabolic changes would account for the above quantitative differences observed in response to the same dose of drug. Also, as discussed in the previous section, repeated administration of PTZ may give rise to an adaptation of the animal to any drug induced peripheral cues such as autonomic activation and thereby result in a shorter lasting, more specific drug stimulus.

Table 5.4 (a) The time of response of pigs C1, C3, C4 and C5 on the PTZ appropriate lever following an injection of the training dose of PTZ in the training sessions up to satisfaction of the discrimination criterion.

Pig number	PTZ dose (mg/kg, i.v.)	Number of sessions	Time of response on drug lever (sec; Mean ± S.E.M.)	PTZ Lever
C1	8.75	5	121.3 ± 4.1	A
C3	7.185	5	288.3 ± 3.9	A
C4	3.5 3.2	1 4	188.4 176.1 ± 3.7	B
C5	10	5	410.7 ± 71.3	A

Table 5.4 (b) The time of response of pigs C1, C3, C4 and C5 on the PTZ appropriate lever following an injection of the training dose of PTZ in successive training sessions after the criterion of successful discrimination was satisfied.

Pig number	PTZ dose (mg/kg,i.v.)	Number of sessions	Time of response on drug lever (sec; Mean ± S.E.M.)
C1	8.75	8	171.0 ± 3.1
	8.44	1	191.1
	8.13	1	180.7
	7.8	1	192.3
	7.5	2	181.1 ± 2.2
	7.2	1	172.4
	6.9	1	168.9
	6.6	8	176.4 ± 4.1
	6.3	1	175.1
	6.0	1	178.7
	5.4	19	149.4 ± 10.8
	5.5	3	94.3 ± 9.4
C3	7.185	2	288.4 ± 0.2
	6.56	12	268.5 ± 15.9
	6.25	13	168.5 ± 6.2
	5.0	27	154.5 ± 6.6
	5.313	8	154.3 ± 21.7
	5.0	2	111.5 ± 12.2
	5.313	2	118.9 ± 14.2
	5.256	9	98.8 ± 9.5

Table 5.4 (b) contd.

C4	3.2	6	155.4 ± 13.5
	3.35	2	55.4 ± 21.3
	3.425	13	102.5 ± 6.4
	2.8	5	98.4 ± 7.0
	2.95	5	90.1 ± 21.3
	3.025	2	77.6 ± 31.0
	3.1	1	36.7
	2.8	11	59.6 ± 9.3
C5	8.75	3	291.5 ± 1.4
	7.5	1	288
	6.875	2	289 ± 10.6
	6.25	28	222.7 ± 11.9
	5.94	3	184.1 ± 10.2
	5.6	1	188.7
	5.3	2	178.3 ± 0.3
	5.0	8	142.6 ± 12.9
	4.375	8	108.4 ± 6.3
	5.0	16	93.0 ± 8.4

The mean frequencies at which the animals pressed the levers, following saline or PTZ treatments in the 10 sessions up to satisfaction of criterion are shown in Table 5.5. There was no significant difference between frequencies on lever A and lever B following saline treatment for pigs C1, C4 and C5. For pig C3 the frequency of response on lever A was significantly ($P<0.0001$) greater than on lever B, following saline treatment. The frequency of response on lever A was significantly ($P<0.01$) greater during saline sessions than during PTZ sessions for this pig but frequencies of response on the drug designated lever did not vary significantly between saline and PTZ sessions for the other animals.

Table 5.5 Frequency of lever pressing for pigs C1, C3, C4 and C5 following an intravenous injection of saline or the training dose of PTZ in the training sessions up to satisfaction of the discrimination criterion. * $P < 0.01$ **v** PTZ lever, # $P < 0.0001$ **v** Lever B; Two sample t test.

Pig number	C1			C3		
Treatment	SALINE		PTZ	SALINE		PTZ
Lever	A	B	A	A	B	A
Frequency of lever presses (Mean ± S.E.M.)	3.3 ± .5	3.2 ± .1	3.2 ± .1	4.0 ±.0 * #	3.4 ± .0	3.8 ± .0
Pig number	C4			C5		
Treatment	SALINE		PTZ	SALINE		PTZ
Lever	A	B	B	A	B	A
Frequency of lever presses (Mean ± S.E.M.)	2.9 ± .4	3.1 ± .1	3.1 ± .1	3.1 ± .1	3.1 ± .1	3.2 ± .1

Fig 5.2 shows a cumulative response record from pig C1 following treatment with saline or PTZ. This record is typical of saline and PTZ training sessions within criterion for all of the pigs with the only exception being the time of response on the drug designated lever following treatment with PTZ, which varied for each pig as shown in Table 5.4.

When pigs were pharmacologically conditioned by the conventional method (Section 5.2.a), only 2 out of 5 animals satisfied the discrimination criterion. In contrast, all of the pigs conditioned by the novel procedure described in this section learned to discriminate the effect of PTZ from saline. One reason for the success of this procedure maybe that the animals are required to emit only one type of response in the undrugged condition i.e. alternation of lever selection. This response is the same for both the initial training period when the animals are untreated and for pharmacological conditioning following saline treatment. On treatment with PTZ the animals are trained to associate the PTZ cue with selection of one lever only. Pharmacological conditioning, therefore, requires a distinction to be made between a newly learned response and a response which has always been associated with the undrugged condition. In contrast, the conventional pharmacological conditioning procedure requires the animal to associate the PTZ or saline condition with either of two levers, both of which, were previously associated with the undrugged condition. Quite apart from the difficulty that this method might impose on the acquisition of the discriminative response, there is also a risk that the animal may develop a lever preference prior to pharmacological conditioning. This problem was overcome to some extent in the present study by including a period of training whereby the reinforced lever was alternated between sessions (see Chapter 2 Section 2.3.d, Procedure 2A). Lever bias also poses a problem when rats are conditioned by this method and such lever bias may influence the determination of ED_{50} values for the ability of drugs to mimic or antagonise the stimulus induced by the training drug (Harris et al., 1988).

From the above it is clear that the novel PTZ conditioning procedure has many advantages over the conventional training method. The pilot study conducted using a tone stimulus revealed the potential of this procedure to detect both the onset and offset of the discriminative stimulus within a single test session. The results described above confirm this finding to some extent, in that PTZ treated animals showed a tendency to respond on the alternate lever after a period of response on the drug lever alone. A more thorough investigation of the ability of this test to detect a change in the psychological state of the animal was conducted and the results are presented below.

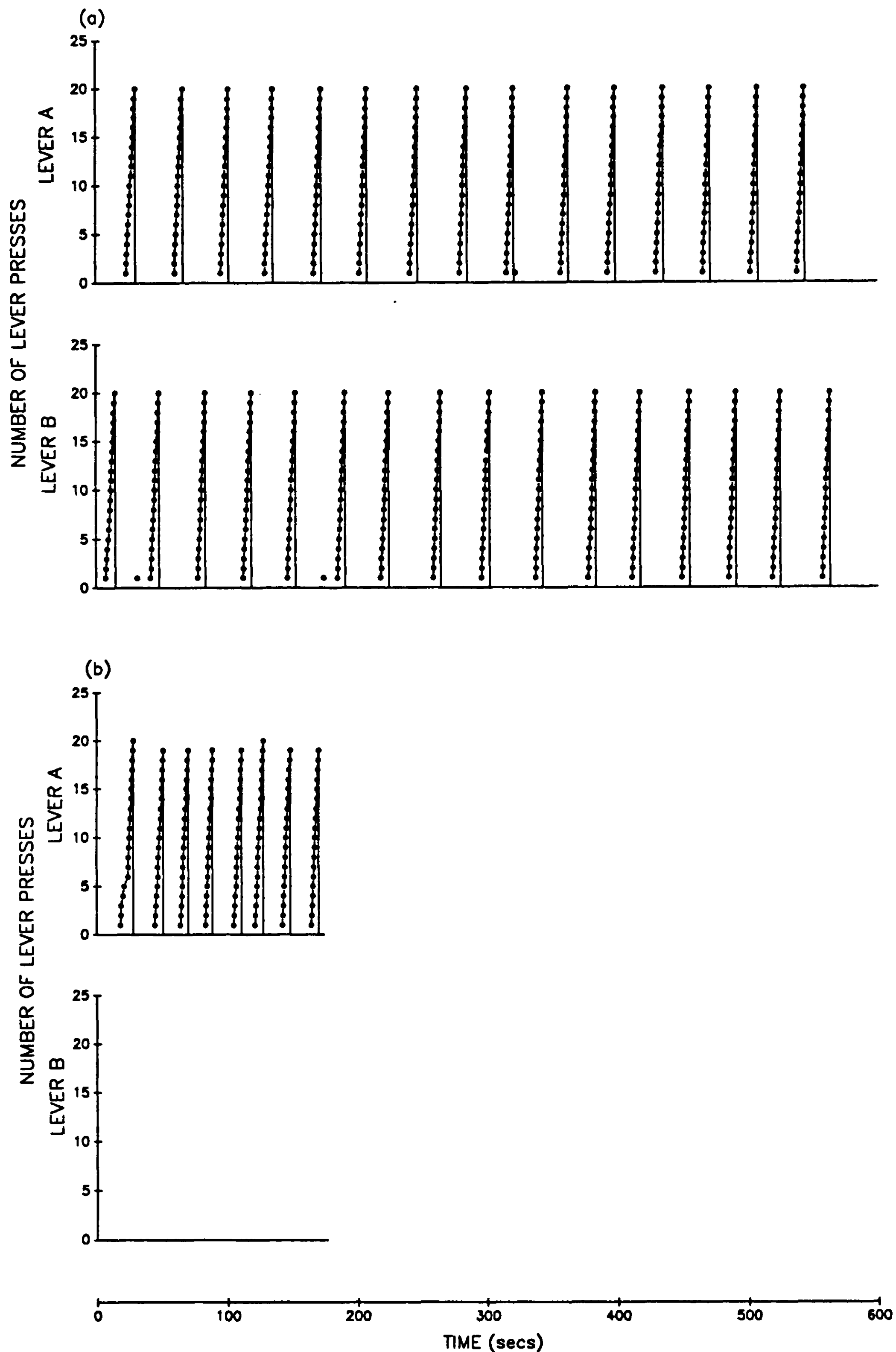


Fig. 5.2 Cumulative records from a saline and PTZ training session administered to pig C1: (a) an alternation of lever selection response to a saline pretreatment, (b) response on the PTZ appropriate lever following an injection of the training dose of PTZ (8.75 mg/kg, i.v.)

The detection of changes in psychological state

Following a saline pretreatment, pigs were tested and after 5 mins of a correct alternation of lever selection response, the training dose of PTZ or the same volume of saline was presented by remote intravenous infusion (Chapter 2, Section 2.3.e). The cumulative records obtained from these tests are shown in Figures 5.3 to 5.6. Following infusion of saline, all of the animals continued to select both levers alternately. Infusion of the training dose of PTZ (8.75 mg/kg) to pig C1 produced an exclusive selection of the drug designated lever for the entire 5 min test period, following the infusion (Fig 5.3b). Likewise, presentation of 7.185 mg/kg PTZ to pig C3 produced selections of the drug designated lever only for the remainder of the test period (Fig 5.4b). Infusion of the training dose of PTZ (6.25 mg/kg) to pig C5 resulted in an initial period of alternation responding. The final press on Lever B occurred 61.9 seconds following the drug infusion and thereafter this animal selected the drug designated lever only (Fig 5.5b). When pig C4 was infused with 3.425 mg/kg PTZ, selections of the drug designated lever only occurred for a period of 150.2 seconds. The animal then began a selection of the alternate lever which was followed by an alternation of lever selection response for the remainder of the test period (Fig. 5.6b). This time of drug lever selection compares well with the mean time of response (102.5 secs) on the drug designated lever estimated from the PTZ training sessions conducted at this stage of the experiment with the same dose of PTZ (Table 5.4). The difference between the PTZ lever response times calculated from the above tests and those estimated from training sessions reflects the variables encountered in the training procedure, such as the time delay in getting the pig into the Skinner box. For all four pigs, the response to an infusion of PTZ during a saline session was significantly ($P<0.05$) different from the response to an infusion of saline.

Further test sessions of PTZ infusion during a saline session, as described above, were carried out on each pig during the course of subsequent PTZ and saline training sessions. The results from these tests are presented in Fig 5.7. Consistent with the findings reported above (Table 5.4) for response times on the PTZ lever as a function of training dose of PTZ, there was a tendency for the duration of these responses to decrease as the dose of the drug

was decreased. However, for pig C1, infusion of a dose of 4.15 mg/kg produced a response of longer duration than later infusions of 5.5 mg/kg. Again, this may reflect an increased specificity of the PTZ cue with repeated administration of the drug or a PTZ induced change in its own metabolism. Such explanations might also account for some of the variation observed in drug lever response times for individual pigs, between consecutive sessions employing the same dose of PTZ (Fig 5.7). However, the failure to observe a consistent decrease in such response times, rules against this possibility. A more plausible explanation for these variations might be subtle changes in the baseline state of the animal which cause the effect of PTZ to be enhanced.

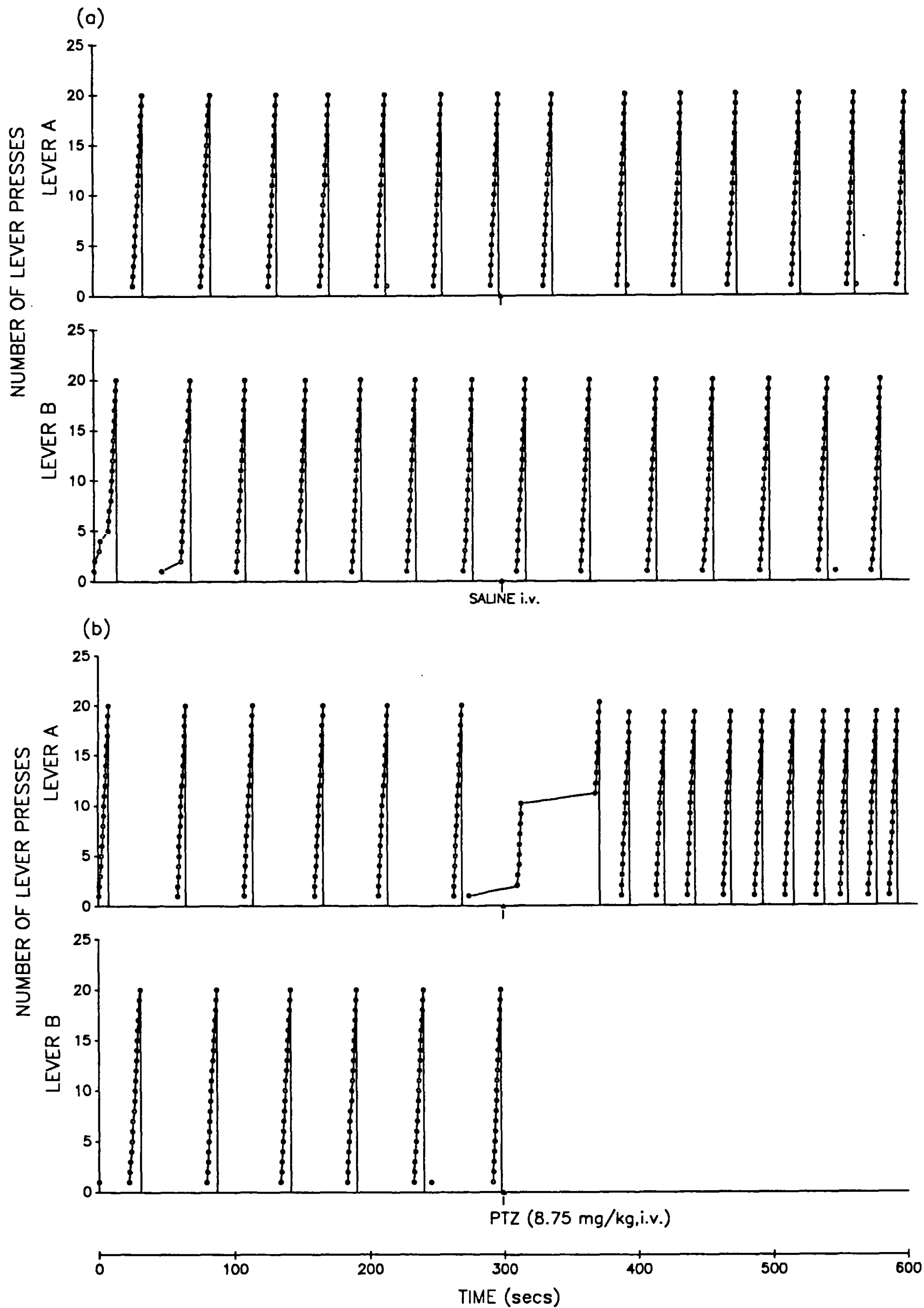


Fig. 5.3 The discriminatory response of pig C1 to an intravenous infusion of saline or PTZ 300 secs after an alternation of lever selection response to a saline pretreatment: (a) an infusion of saline, (b) an infusion of the training dose of PTZ (8.75 mg/kg).

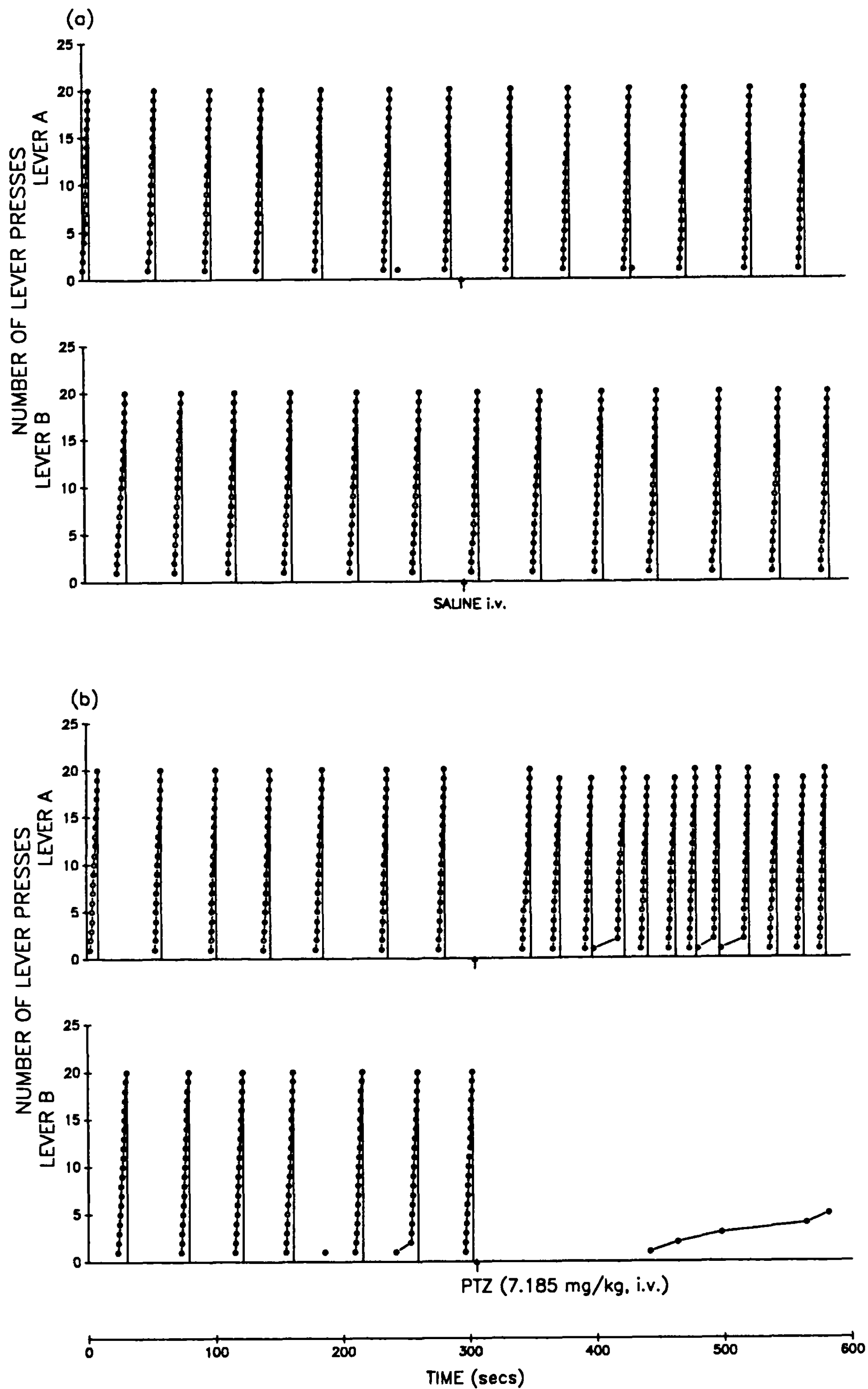


Fig. 5.4 The discriminatory response of pig C3 to an intravenous infusion of saline or PTZ 300 secs after an alternation of lever selection response to a saline pretreatment: (a) an infusion of saline, (b) an infusion of the training dose of PTZ (7.185 mg/kg).

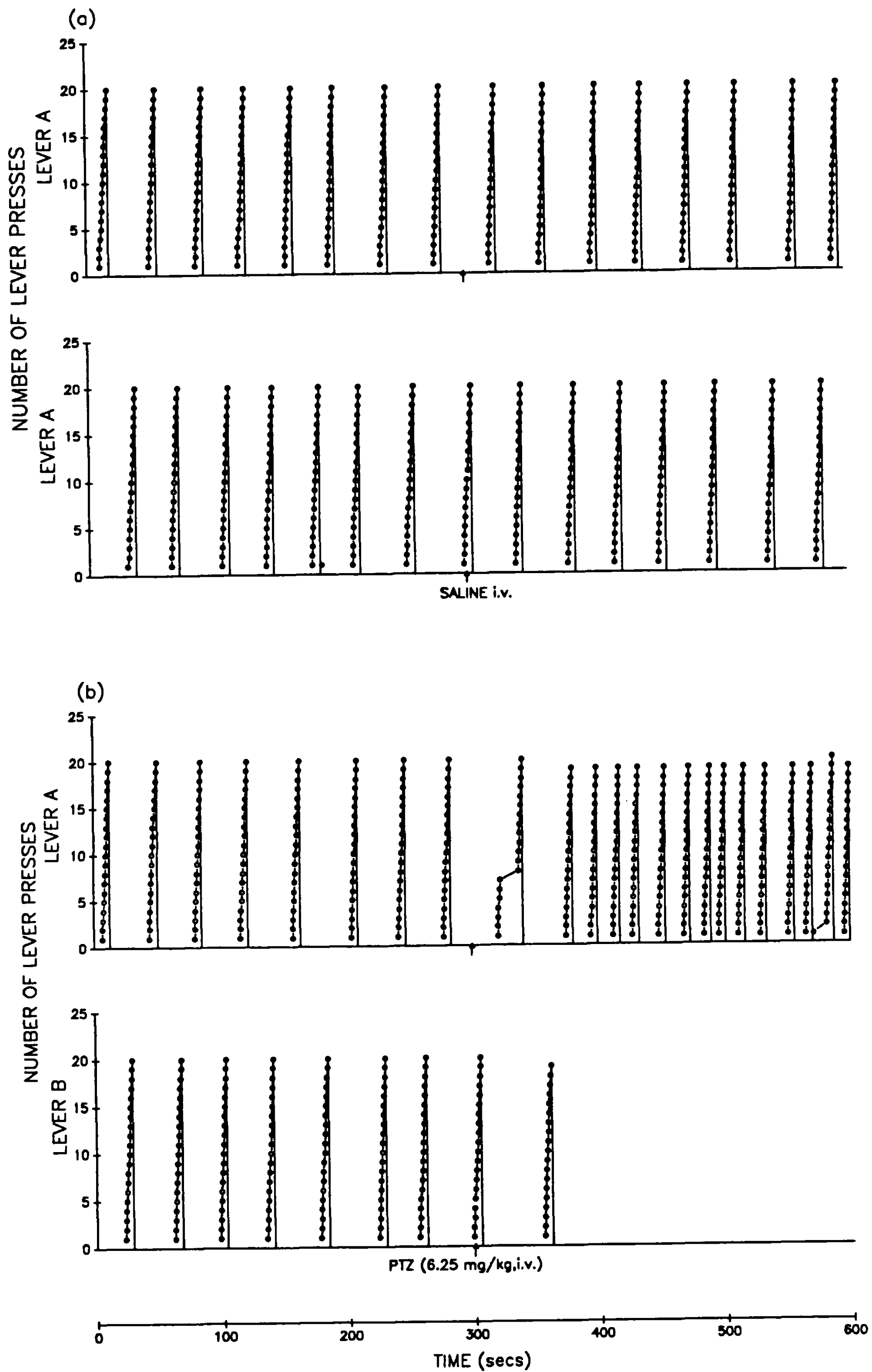


Fig. 5.5 The discriminatory response of pig C5 to an intravenous infusion of saline or PTZ 300 secs after an alternation of lever selection response to a saline pretreatment: (a) an infusion of saline, (b) an infusion of the training dose of PTZ (6.25 mg/kg).

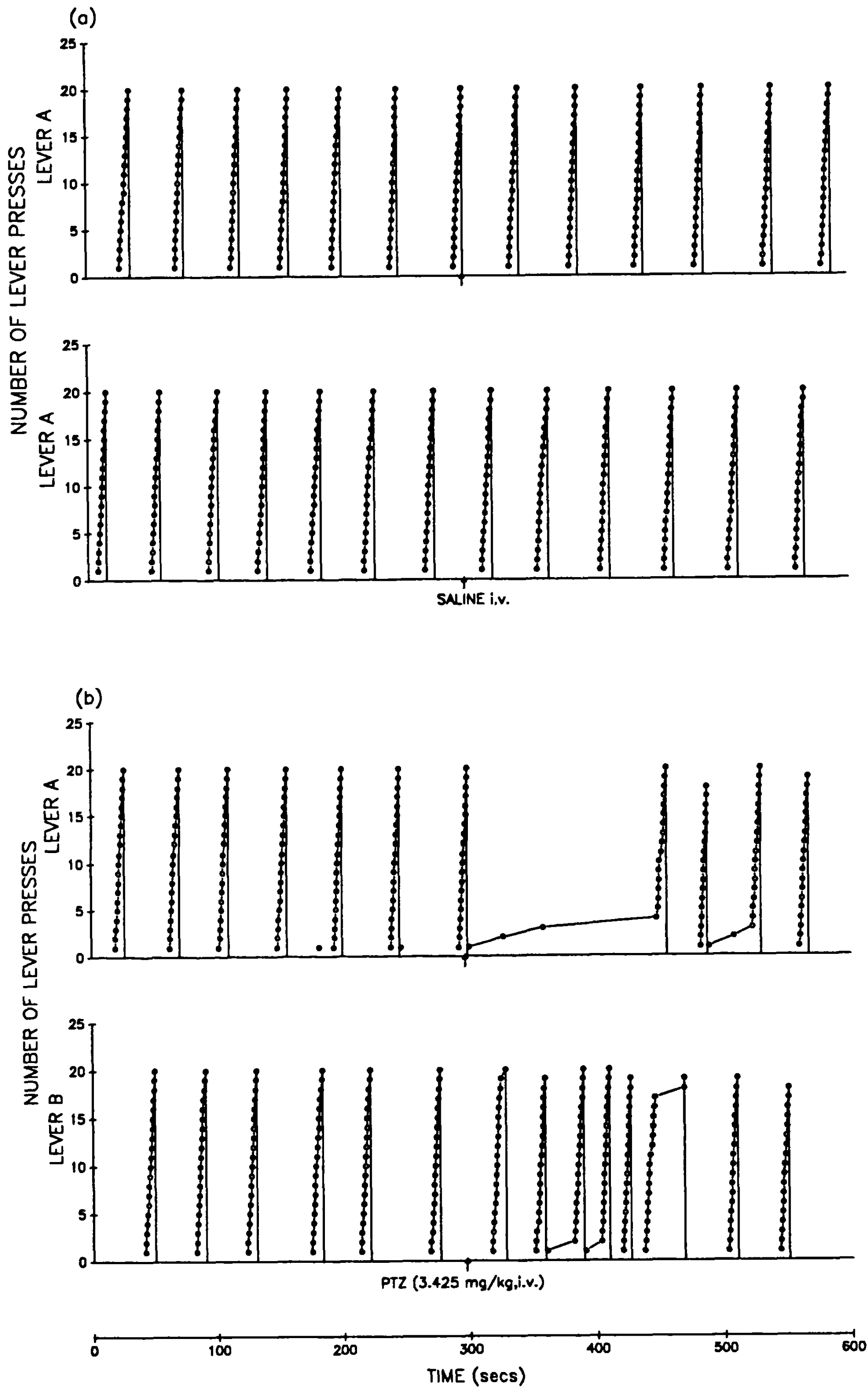
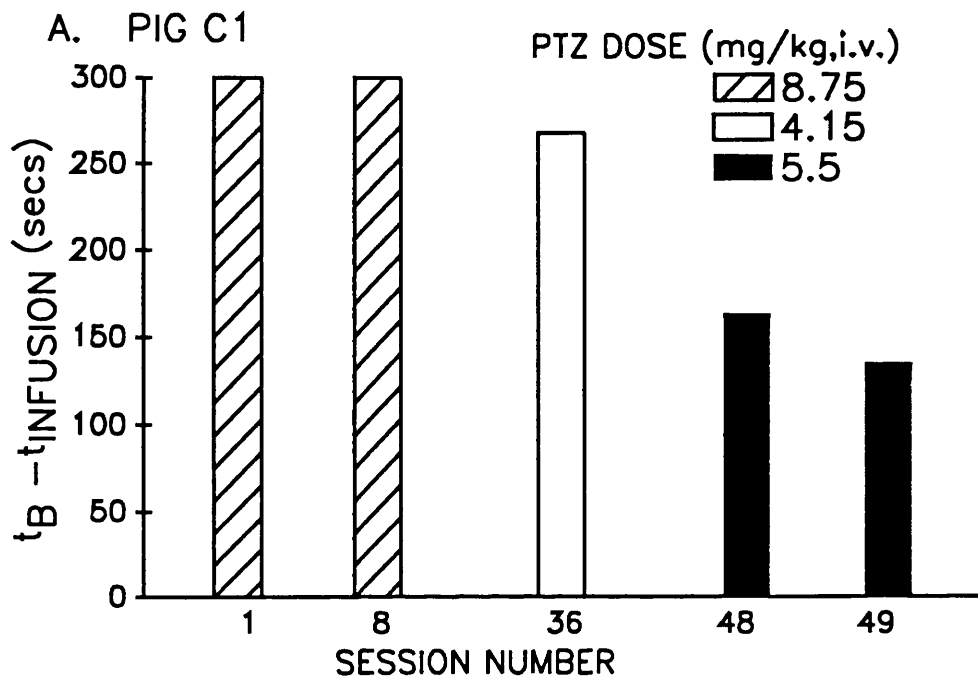
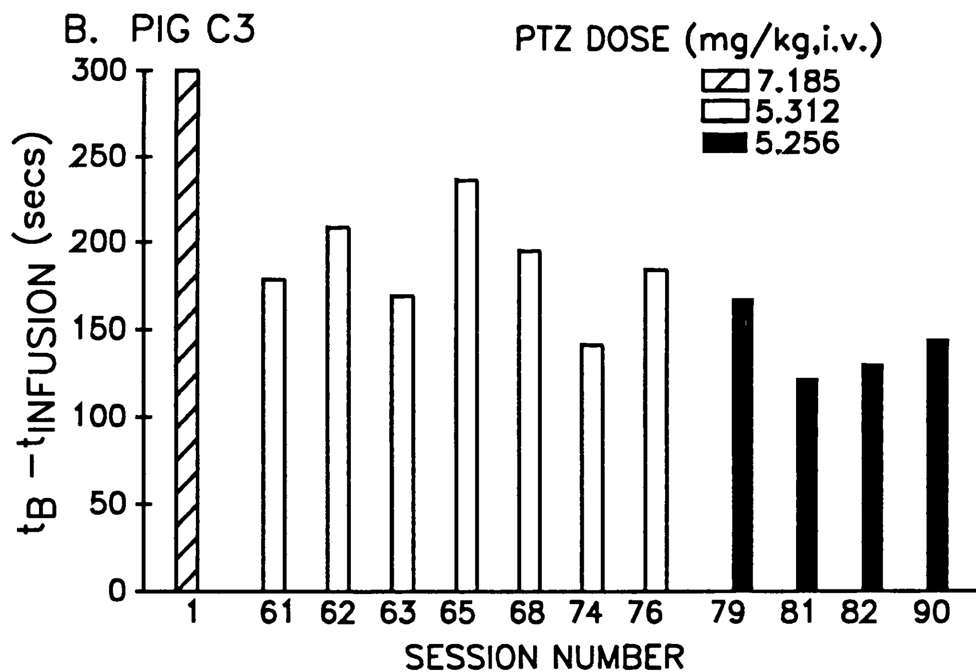
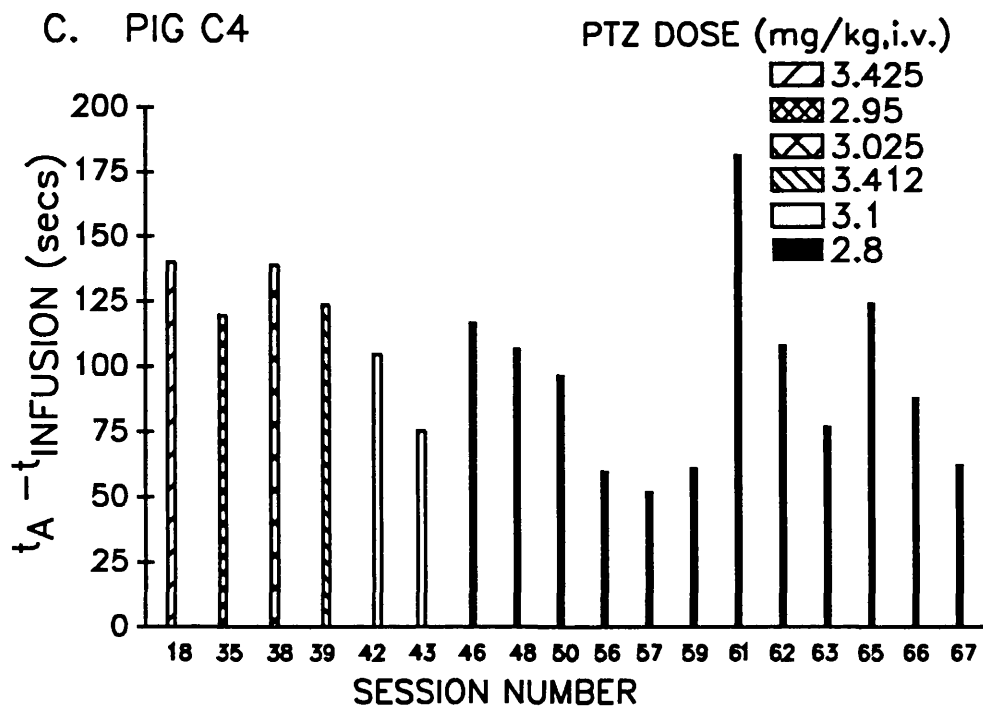


Fig. 5.6 The discriminatory response of pig C4 to an intravenous infusion of saline or PTZ 300 secs after an alternation of lever selection response to a saline pretreatment: (a) an infusion of saline, (b) an infusion of the training dose of PTZ (3.425 mg/kg).


A. PIG C1
PTZ DOSE (mg/kg,i.v.)
8.75
4.15
5.5
300
250
200
150
100
50
0
$t_B - t_{INFUSION}$ (secs)
1
8
36
48
49
SESSION NUMBER


B. PIG C3
PTZ DOSE (mg/kg,i.v.)
7.185
5.312
5.256
300
250
200
150
100
50
0
$t_B - t_{INFUSION}$ (secs)
1
61
62
63
65
68
74
76
79
81
82
90
SESSION NUMBER



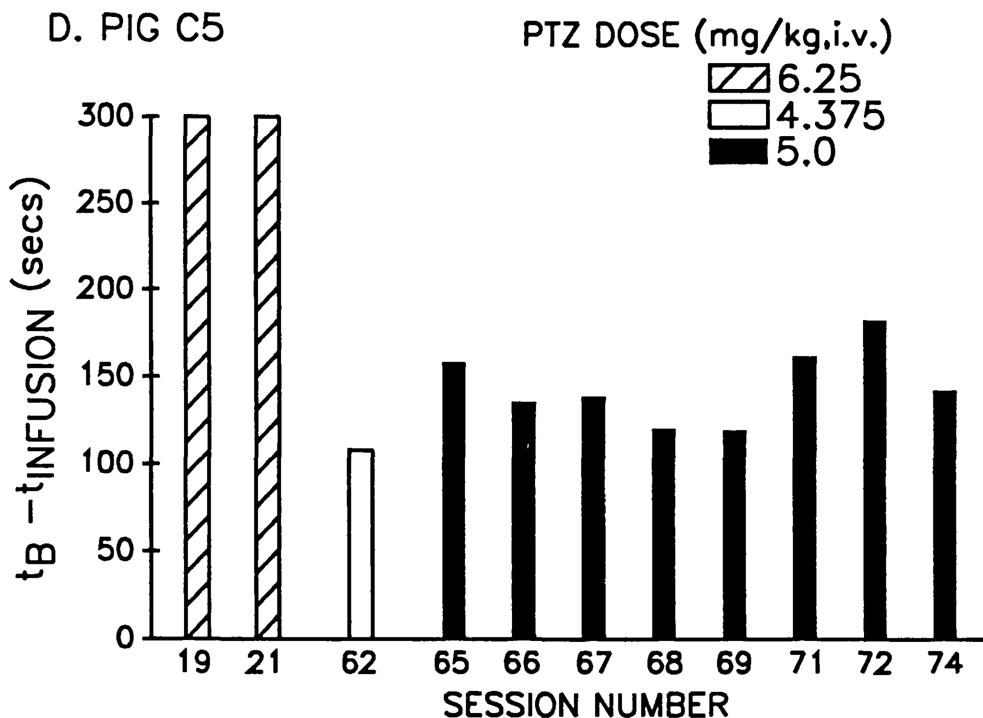


Fig 5.7 The time of response for pigs C1 (A), C3 (B), C4 (C) and C5 (D) on the PTZ appropriate lever as a function of PTZ dose and the frequency of PTZ treatments (session number) following the satisfaction of the discrimination criterion. The time of response on the PTZ appropriate lever represents the time from an intravenous infusion of PTZ during a saline session to a start of a selection of the alternate lever followed by an alternation of lever selection response ($t_{A/B}$ -$t_{INFUSION}$).

5.3 Conclusions

The results from the present study show that pigs can be conditioned to discriminate the effects of a subconvulsant dose of PTZ from saline. This detection of an interoceptive discriminative stimulus property of PTZ is in agreement with other studies using the conventional drug discrimination paradigm, whereby animals are trained to associate the drug stimulus with one lever and the drug vehicle condition with the other lever (eg. Shearman & Lal, 1979, 1980; Stephens et al., 1984; Vellucci et al., 1988; Ator et al., 1989). However, results from the present study demonstrate that an investigation of the PTZ cue by the conventional conditioning procedure is subject to a major limitation in that only those responses before first reinforcement can be accepted as a valid index of the PTZ stimulus. Responses on the PTZ lever following initial reinforcement may reflect a fixation on this lever rather than an association with the drug cue. A preliminary experiment revealed that the same situation arises following initial reinforcement on the saline lever where the animal continued to select this lever after application of the PTZ stimulus. Such a fixation on the lever initially reinforced also occurs in rats which are trained by this procedure (see Lal & Emmett-Oglesby, 1983).

A conditioning procedure was developed which enabled a study of the PTZ cue in pigs without the limitations imposed by the conventional conditioning paradigm. Pigs were trained to select both levers alternately in the undrugged condition and to select one lever only on administration of PTZ. This discrimination paradigm proved more successful than the conventional procedure, as judged by the number of animals which satisfied the set discrimination criterion. Moreover, the novel conditioning procedure enabled the onset and offset of the PTZ stimulus to be determined within a single test session. Since this procedure is sensitive to the detection of changes in the psychological state of the animal, it introduces the possibility of evaluating the anxiogenic nature of the PTZ stimulus by examination of the discriminatory response of animals to the state induced by a conditioned anxiogenic stimulus. Such an evaluation would not have been possible using the conventional PTZ discrimination paradigm since the induction and assessment of a conditioned

emotional response requires a stable behavioural baseline, prior to the application of the conditioned stimulus. The ability to monitor a change in the response of the pig from the undrugged condition to the drugged condition enables the pre-drug response period to be used as a behavioural baseline to examine any non-specific effects unrelated to drug action. This procedure, therefore, also should facilitate a specific and precise pharmacological study of the PTZ stimulus since the time course of a drug can be monitored within a single test session in addition to any non-specific effects of such drugs on the discriminatory behaviour of the animals.

Chapter 6

A behavioural and pharmacological evaluation of the discriminative stimulus induced by PTZ in the pig.

6.1 Introduction

6.2 Results and discussion

6.2.a An investigation of the discriminatory response of pigs to the state induced by a conditioned anxiogenic stimulus

6.2.b A pharmacological evaluation of the PTZ stimulus

6.3 Conclusions

6.1 Introduction

While pharmacological evaluations of the pentylenetetrazole (PTZ) discriminative stimulus in rats (Shearman & Lal, 1979, 1980; Stephens et al., 1984; Vellucci et al., 1988) have revealed that the stimulus is sensitive to modulation by drugs which affect $GABA_A$ receptor function, they do not provide conclusive evidence that the PTZ cue corresponds to a state of anxiety since agents which mimic or antagonise the cue also possess proconvulsant or anticonvulsant properties, respectively. Such evidence could be obtained from a behavioural investigation of the PTZ cue, by comparing a conditioned emotional response (CER) to the PTZ injected state. The CER paradigm represents an animal model of anticipatory anxiety (Estes & Skinner, 1941). Neurochemical investigations have revealed that the CER in rats is modulated by a GABAergic mechanism (Lane et al., 1982a, b). These authors have shown that the conditioned emotional state (CES) in rats is associated with a decreased utilisation of GABA in certain limbic areas and also a decrease in benzodiazepine binding in the cerebral cortex. In addition, several studies have demonstrated that the CES in rats is sensitive to alleviation by benzodiazepine minor tranquillisers (Lauener, 1963; Tenen, 1967; Lane et al., 1982a, b).

There are no reports of attempts to conduct such an evaluation of the PTZ cue in rats and this may reflect the limitations imposed by the pharmacological conditioning procedure employed to study the PTZ discriminative stimulus in these animals (see Chapter 5, Section 5.2.a). In Chapter 5, the development of a novel conditioning procedure in the pig was described. This procedure overcame the major limitation of the conventional method which allows a study of the PTZ cue only during the response period before first reinforcement. The suitability of the novel conditioning procedure for the above behavioural investigation has been discussed (Chapter 5, Section 5.2.a and 5.3).

The aim of the present study, therefore, was to investigate the anxiogenic nature of the PTZ stimulus in pigs by examining the discriminatory response of these animals to the state induced by a conditioned anxiogenic

stimulus. An investigation of the pharmacological specificity of the PTZ stimulus was also conducted.

6.2 Results and discussion

6.2.a An investigation of the discriminatory response of pigs to the state induced by a conditioned anxiogenic stimulus.

A CES was induced in three of the pigs which were trained to discriminate PTZ from saline by the novel conditioning procedure described in the previous Chapter (and see Chapter 2, Section 2.3.d, Procedure 2B for details of the conditioning procedure). A tone stimulus was presented to each animal on at least two occasions before it was conditioned to ensure that this stimulus was neutral i.e. did not disrupt the alternation of lever selection response to a saline pretreatment. The tone stimulus was then paired with a mild electric shock in a situation distinct from operant training. The effect of the conditioned stimulus (CS) on the discriminatory response of the animals was examined by presentation of the CS for a duration of 3 min beginning 5 min after the start of a saline session. The results obtained from this study are summarised in Table 6.1.

Generalisation of the CES to the PTZ cue.

When the neutral tone stimulus was applied during a saline session, the animals continued to select both levers alternately (Figs 6.1.a, 6.2.a and 6.3.a). Following the first pairing of the tone stimulus with shock, presentation of the CS during a saline session did not affect the alternation of lever selection response of pig C3 (Fig 6.1.b). During the first application of the CS, pig C1 made two consecutive selections of the drug designated lever (Fig 6.2.b). However, the overall response during the CS period did not differ significantly from that observed during the period of neutral tone stimulus application (Fig 6.2.a). The first presentation of the CS to pig C5 resulted in two occurrences of two consecutive selections of the B lever (see Table 6.1). This result clearly

shows a disruption of the typical response to a saline pretreatment but it does not represent a state analogous to that induced by PTZ since the A lever was the drug designated lever for this animal.

Presentation of the CS to pigs C3 and C1, following a second pairing with shock, resulted in a switch in response from alternation of lever selection to selection of the drug designated lever alone. After a period of 48.8 secs, the response of pig C3 consisted of selections of the drug designated lever and this behaviour persisted until the CS was removed when the animal again selected both levers alternately as for the pre-CS period (Fig. 6.1.c). The response during the CS period was significantly ($P<0.02$) different from the alternation of lever selection response observed during the application of the neutral tone stimulus (Fig 6.1.a) and during the first application of the CS (Fig 6.1.b). At 87.1 secs after the onset of the CS, pig C1 began a selection of the drug designated lever which was followed by a period of response exclusive to this lever (Fig 6.2.c). A recovery of an alternation of lever selection response was not observed in this animal until 166.7 secs after the offset of the CS. The response during and after the application of the CS was significantly ($P<0.05$) different from that observed on presentation of the neutral tone stimulus (Fig 6.2.a).

Presentation of the CS to pig C5, following the second tone/shock pairing resulted in two consecutive selections of the drug designated lever (Fig 6.3.b). Although the animal then continued to select both levers alternately, a suppression of response occurred for a period of 66.1 secs before and 55.5 secs after the offset of the CS. Although this animal did not receive any additional tone/shock pairings, the next presentation of the CS resulted in total suppression of response during the CS period (Fig 6.3.c). Following the offset of the CS the animal made two consecutive selections of the drug designated lever and this was followed by an alternation of lever selection response for the remainder of the test period. The next presentation of the CS produced a change in response from alternation of lever selection to selection of the drug designated lever alone (Fig 6.3.d). The animal continued to select this lever for a period of 44 secs after the offset of the CS. During presentation of the CS a

suppression of response occurred for a period of 103.7 secs. The response during the CS period was significantly ($P<0.05$) different to that observed on application of the neutral tone stimulus.

Application of the CS to pig C3, following the third tone/shock pairing, resulted in a suppression of response during the CS period (Fig 6.1.d). During the period of CS presentation only two lever selections were made. Although, the response during the CS period consisted of an alternate selection of both levers, the initial selection of the drug designated lever represents a disruption of an alternation response since this was the last lever selected before CS presentation. Presentation of the CS to this pig in the absence of further tone/shock conditioning, resulted in a reversal of the suppression of response observed during the CS period (Fig 6.1.e-f) and the response to the CS then consisted of alternation of lever selections like that observed on application of the neutral tone stimulus. This animal, then, received several such extinction sessions. When it was clear that the tone stimulus did not induce any disruption of the alternation response (Fig 6.4.a), this stimulus was again conditioned by pairing it with mild electric shock in a situation distinct from operant conditioning. Following the first tone/shock pairing, application of the CS during a saline session produced a disruption of alternation of lever selection and the animal made two consecutive selections of the drug designated lever before the offset of the CS (Fig 6.4.b). Presentation of the CS during a saline session, following a second pairing of tone and shock resulted in a change in response from alternation of lever selection to selection of the drug designated lever alone (Fig 6.4.c). At 49.1 secs after the onset of the CS, the animal began a selection of lever A and this was followed by a period of selections exclusive to this lever until the CS was removed. Although this response did not differ significantly from that observed on application of the neutral tone stimulus, it is clear from Fig. 6.4.c that presentation of the CS produced a deviation from an alternation of lever selection response.

Effect of diazepam on the discriminatory response of pigs to the CS

Pigs were treated with diazepam (0.5 mg/kg, p.o.) and tested in the Skinner box 25 min later, after an injection with saline. As before, the CS was presented for 3 min, starting 5 min after the beginning of the test. These test sessions were conducted the day after the above generalisations of the CES to the PTZ cue were observed. The day following the diazepam test, the food vehicle alone was administered to these animals and they were tested with the CS as described above.

Pretreatment of pig C3 with diazepam resulted in an alternation of lever selection response during the CS period (Fig 6.4.d). In contrast, the previous application of the CS produced a deviation from an alternation of lever selection response to a selection of the drug lever alone (Fig 6.4.c). Presentation of the CS to this animal following the administration of the food vehicle alone resulted in a change in response from alternation of lever selection to selection of the drug lever only, with a period of suppressed response for a duration of 92 secs (Fig 6.4.e). These results demonstrate an antagonism of the CES by diazepam. During the test with diazepam, the pig showed a reduced rate of response and made fewer lever selections (Fig 6.4.d) in comparison to the pre-diazepam test (Fig 6.4.c) or the test conducted the day following diazepam administration (Fig 6.4.e). This may reflect a sensitivity of this animal to the sedative action of diazepam, although there were no physical signs of sedation at the time of testing.

To delay the extinction of the CES and thereby facilitate the investigation of the effect of diazepam on this state, the tone stimulus was conditioned in pig C3 after the generalisation to the PTZ cue was observed and again after the test with diazepam. While this protocol allowed a clear demonstration of the antagonistic effect of diazepam on the CES, the animal displayed escape behaviour during subsequent pharmacological conditioning sessions. To avoid the development of this aberrant behaviour in the other two pigs, the tone stimulus was not paired with shock once a generalisation of the CES to the PTZ cue was observed.

Administration of diazepam to pig C1 resulted in an alternation of lever selection response during the CS period (Fig 6.2.d). This result demonstrates a clear antagonism of the generalisation of the CES to the PTZ cue observed on the previous application of the CS to this animal (Fig. 6.2.c). Administration of the food vehicle alone did not produce alternation of lever selections during the CS period. Presentation of the CS resulted in two incidents of two consecutive selections of the drug designated lever, one immediately after the onset of the CS and the other immediately before the offset of this stimulus (Fig. 6.2.e). A subsequent application of the CS produced an alternation of lever selection response, reflecting extinction of the CES (Fig. 6.2.f). These results demonstrate that the alternation of lever selection response after diazepam treatment was due to an antagonistic action of this drug rather than a spontaneous extinction of the CES.

Pretreatment of pig C5 with diazepam resulted in an alternation of lever selection response during the CS period (Fig. 6.3.e). The previous application of the CS to this animal produced a generalisation to the PTZ injected state and also a period of response suppression (Fig. 6.3.d). Administration of the food vehicle alone resulted in a change in response from alternation of lever selection to a selection of the drug designated lever only, during the CS period (Fig 6.3.f). This response was significantly ($P<0.01$) different to that observed after diazepam treatment. It is clear, therefore, that the alternation of lever selection response observed during the CS period after diazepam treatment, is a consequence of a pharmacological action of diazepam and does not reflect a spontaneous extinction of the CES. Additional support for this conclusion comes from the finding that further applications of the CS resulted in generalisation to the PTZ injected state and several such CS presentations were necessary before an extinction of the CES became evident (see Table 6.1). Following the pretreatment of this pig with diazepam, there were two incidents of two consecutive selections of the B lever during the pre-CS period and also 12 extra presses on this lever immediately before the offset of the CS (Fig. 6.3.e). It is clear that the latter responses do not reflect an additional lever selection since the animal did not pause to eat the previous reinforcement. It is unlikely that the additional selections of the B lever during the pre-CS

period reflects an amnesic effect of diazepam since the alternation of lever selection response was otherwise maintained during the 10 min session. However, such behaviour cannot be interpreted as a consequence of the diazepam pretreatment since the response of the animal in the saline training sessions during this period did not satisfy the set discrimination criterion (see below).

Table 6.1 The discriminatory response of pigs to a presentation of a neutral tone stimulus or an aversively conditioned tone stimulus for a duration of 180 secs given 300 secs after a response to a saline (i.v.) or diazepam (0.5 mg/kg,p.o.,25 min) pretreatment. [a] +, tone paired with shock in a non operant situation on the previous evening, - no administration of tone or shock on the previous evening; [b] lever selections for pre-tone period are defined as 5 or more presses on a lever, lever selections for tone and post-tone period when both levers were set to reward are defined as the lever on which reinforcement was obtained; # the post tone period was extended in order to observe a recovery of an alternation of lever selection response. * $P < 0.05$ **v** the corresponding response period from a test with the neutral tone stimulus, ~ $P < 0.05$ **v** the corresponding response period from the previous test with the conditioned stimulus; Fisher Exact test (two-tailed).

Pig number	Drug lever	Treatment	[a] Tone/shock pairing	[b] Lever selections		
				Pre-tone period (300 secs)	Tone period (180 secs)	Post-tone period (120 secs)
C1	A	Saline	Neutral	BABABABABAB	ABABABABA	BABAB
		Saline	+	ABABABAABAB	ABAABAB	ABABA
		Saline	+	ABABABA	BABAAAA *	AAAAAABABAB * #
		Diazepam	-	ABABAABABA	BABABA	BABAB
		Saline	-	ABABAABAB	AABABAA	BABAB
		Saline	-	BABABBABAB	ABABA	BABA

C3	A	Saline	Neutral	ABABABABABABAB	ABABABABAB	ABABABA
		Saline	+	ABABABABABAB	ABABABAB	ABABAB
		Saline	+	ABABABABABAB	ABAAAAA * ~	BABAB
		Saline	+	ABABABABABA	AB	ABABAB
		Saline	-	ABABABABAB	AABABAB	ABABA
		Saline	-	BABABABABABAB	BABABABA	BABABAB
		Saline	Neutral	ABABABABAB	ABABA	BABA
		Saline	+	ABABABAB	ABABAA	BABA
		Saline	+	ABABABA	BAAAA	BABA
		Diazepam	+	BABAB	ABAB	AB
		Saline	+	ABABABAB	AAA	BAA

Table 6.1 contd.

Pig number	Drug lever	Treatment	Tone/shock pairing	Lever selections		
				Pre-tone period	Tone period	Post-tone period
C5	A	Saline	Neutral	BABABABABABABABABA	BABABABABA	BABABAB
		Saline	+	BABABABABABBABAB	ABBABABBA	BABABABA
		Saline	+	ABABABABAABABA	AABAB	AABAB
		Saline	-	ABABABAABABABA		AABABABA
		Saline	-	ABBABABABABABA	BAAA *	AABABAB
		Diazepam	-	BBABABABABB	ABABAB	ABABA
		Saline	-	BABAABABABABA	ABAAAAAA * ~	ABABAA
		Saline	-	BABBABABB	ABAAAAAAAA *	ABABAB
		Saline	-	ABABABABABA	BAAAAAABA *	BABABAB
		Saline	-	ABABABABABAABA	BAABABAAA	BABABA
		Saline	-	ABABAABABABABA	BAAAAAABAA *	ABABAB
		Saline	-	ABABABABABAB	ABABABAB ~	ABABAB

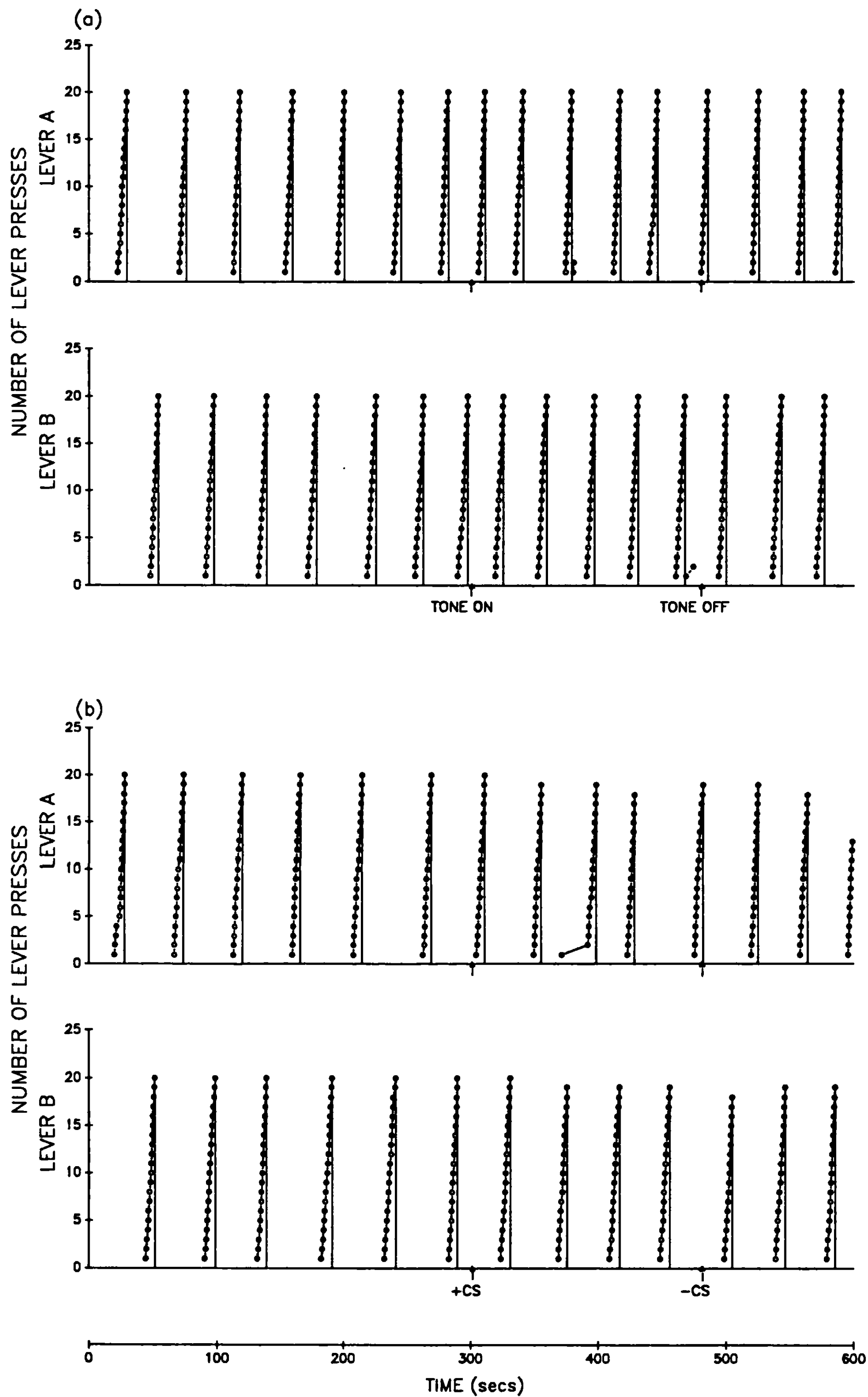


Fig 6.1 Cumulative records of the discriminatory response of pig C3 during the acquisition and extinction of a conditioned emotional state induced by a presentation of an aversively conditioned tone stimulus for a duration of 180 secs starting 300 secs after a response to a saline pretreatment: (a) response to a neutral tone stimulus, (b) response to the tone stimulus following a tone/shock pairing conditioning on the previous evening.

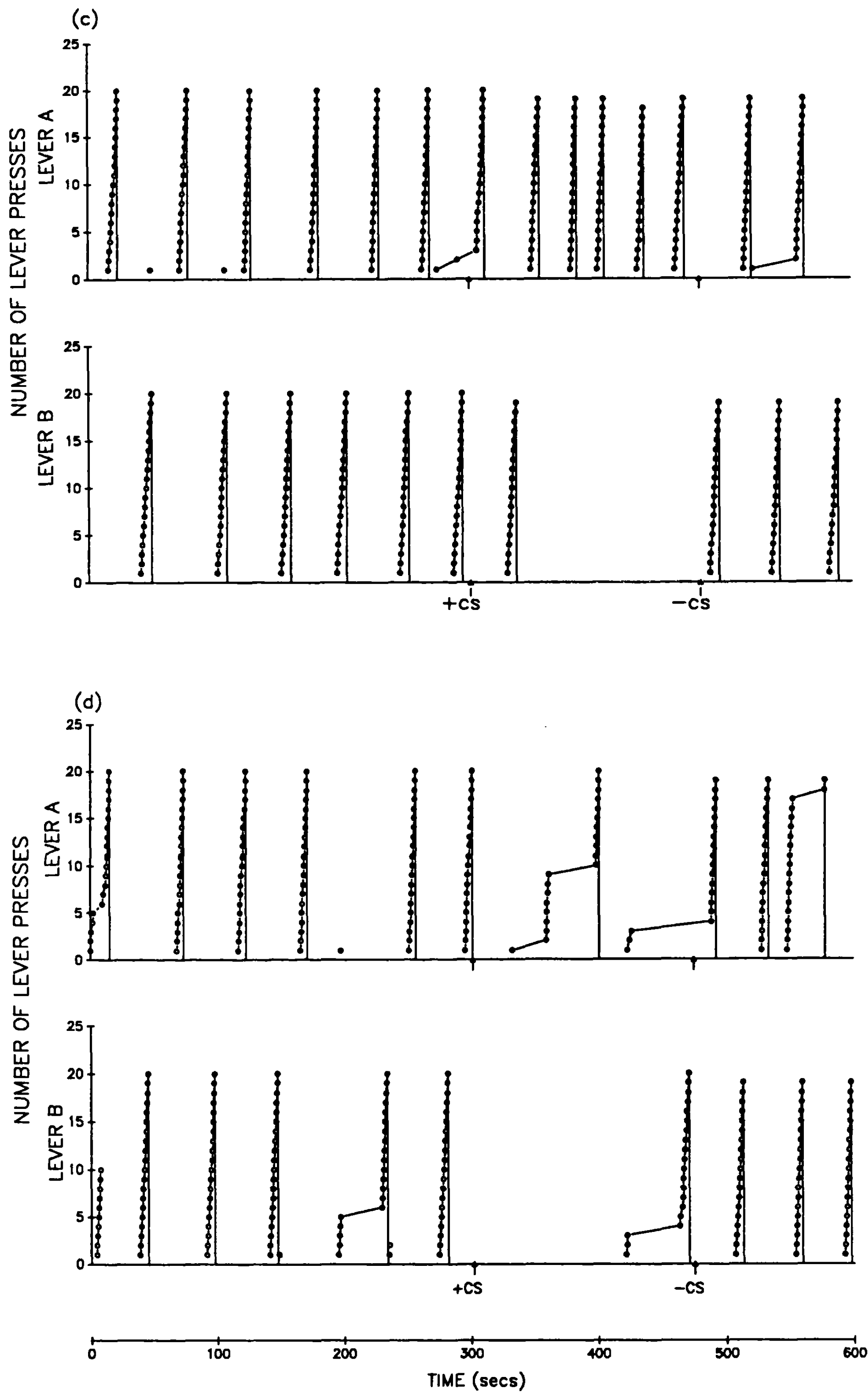


Fig 6.1 contd. (c) response to the conditioned tone stimulus following a second tone/shock pairing conditioning on the previous evening, (d) response to the conditioned tone stimulus following a third tone/shock pairing conditioning on the previous evening.

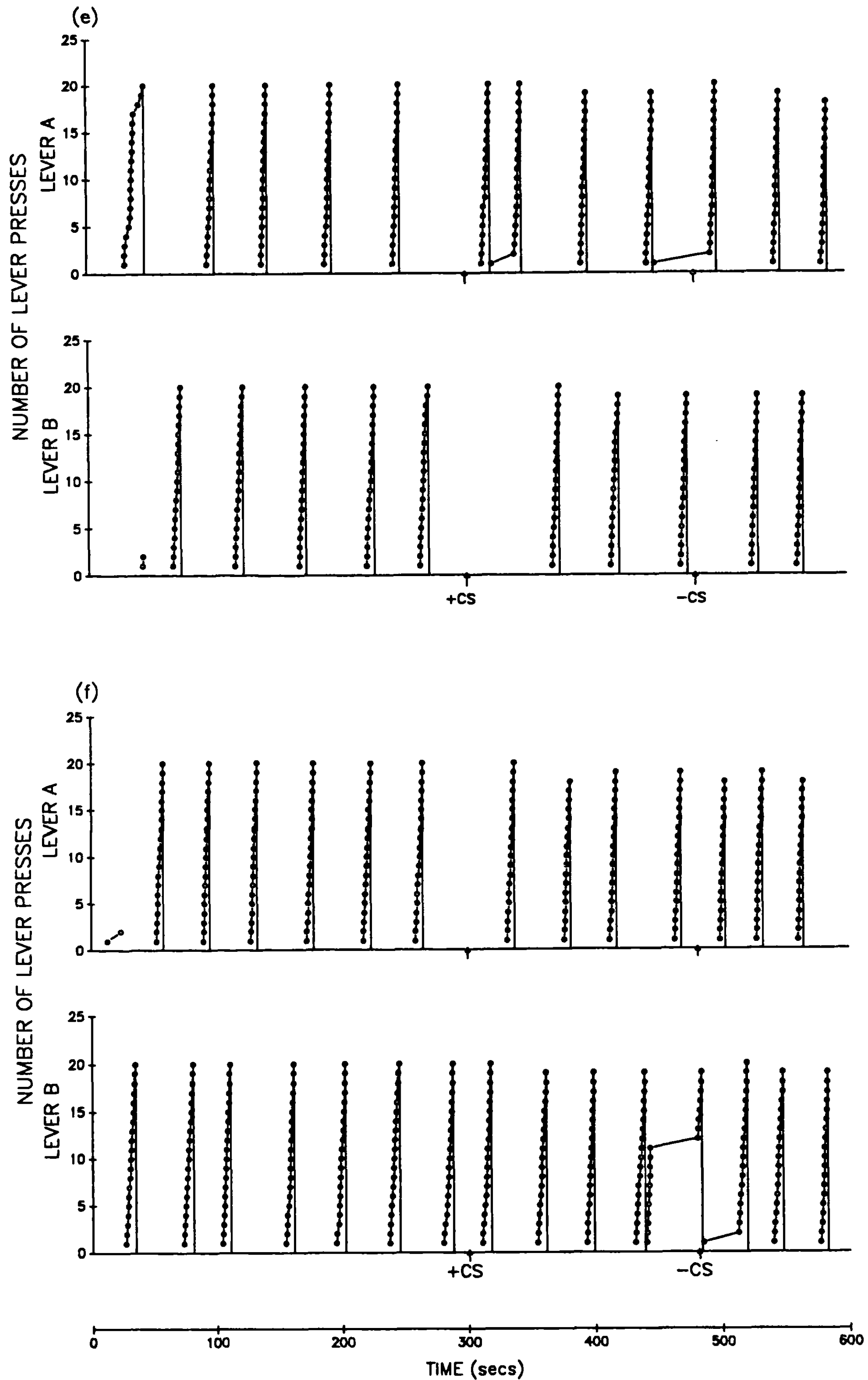


Fig 6.1 contd. (e) response to the conditioned tone stimulus in the absence of a tone/shock pairing conditioning on the previous evening, (f) response to the conditioned tone stimulus in the absence of a tone/shock pairing conditioning on the previous evening.

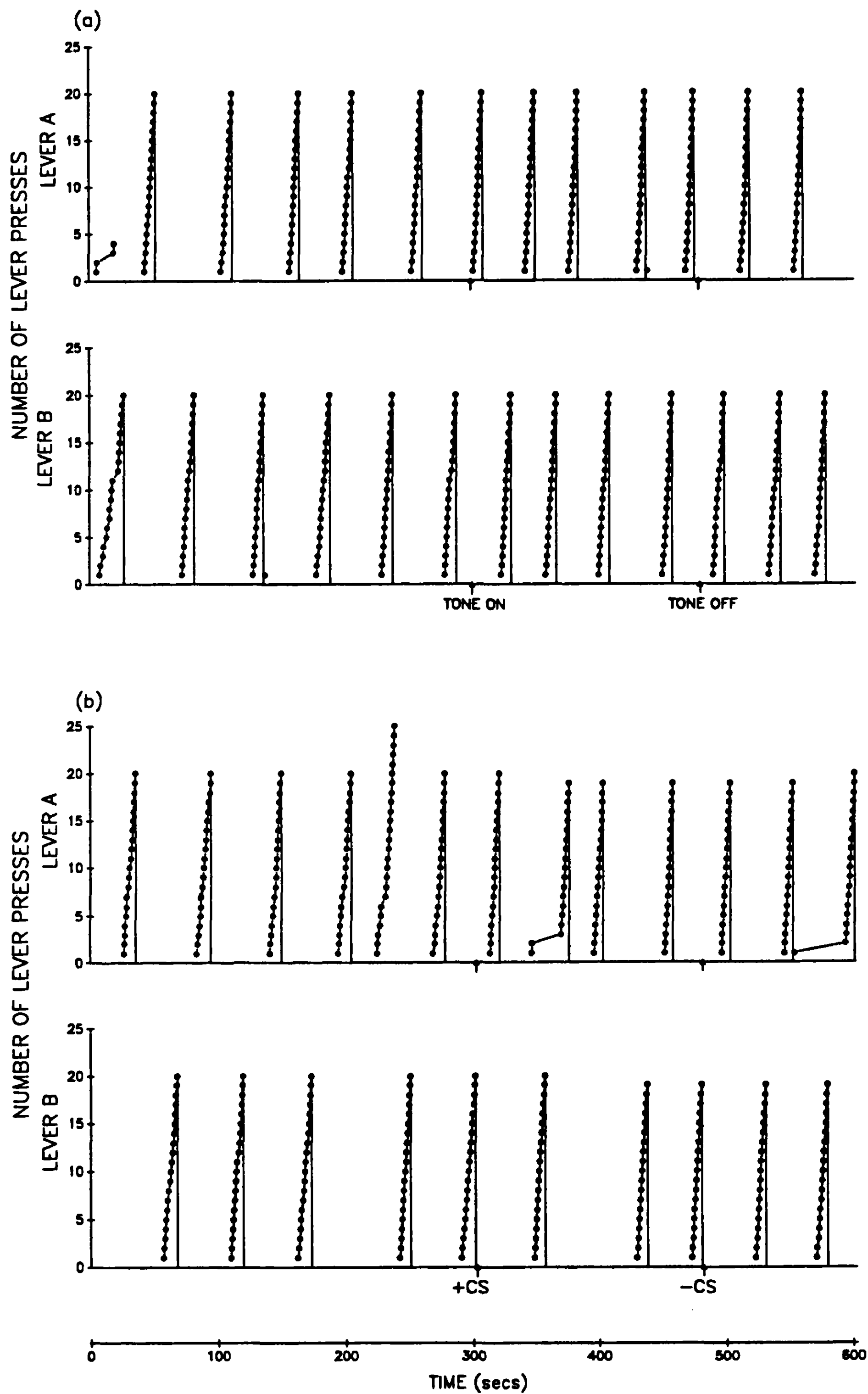


Fig 6.2 Cumulative records of the discriminatory response of pig C1 during the acquisition and extinction of a conditioned emotional state induced by a presentation of an aversively conditioned tone stimulus for a duration of 180 secs starting 300 secs after a response to a saline pretreatment: (a) response to a neutral tone stimulus, (b) response to the tone stimulus following a tone/shock pairing conditioning on the previous evening.

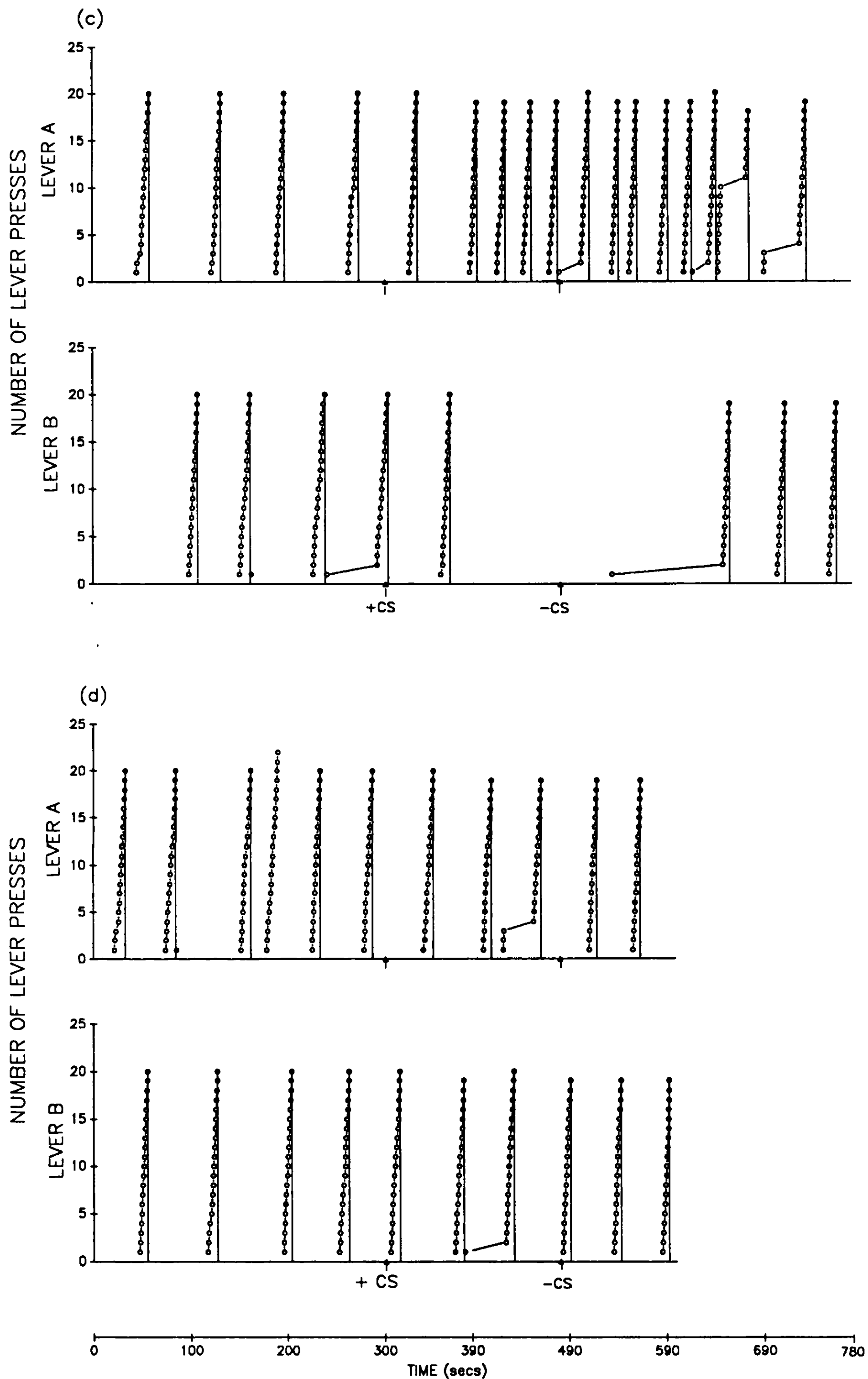


Fig 6.2 contd. (c) response to the conditioned tone stimulus following a second tone/shock pairing conditioning on the previous evening, (d) effect of a diazepam (0.5 mg/kg, p.o., 25 min) pretreatment on the response to the conditioned tone stimulus which was not paired with shock on the previous evening.

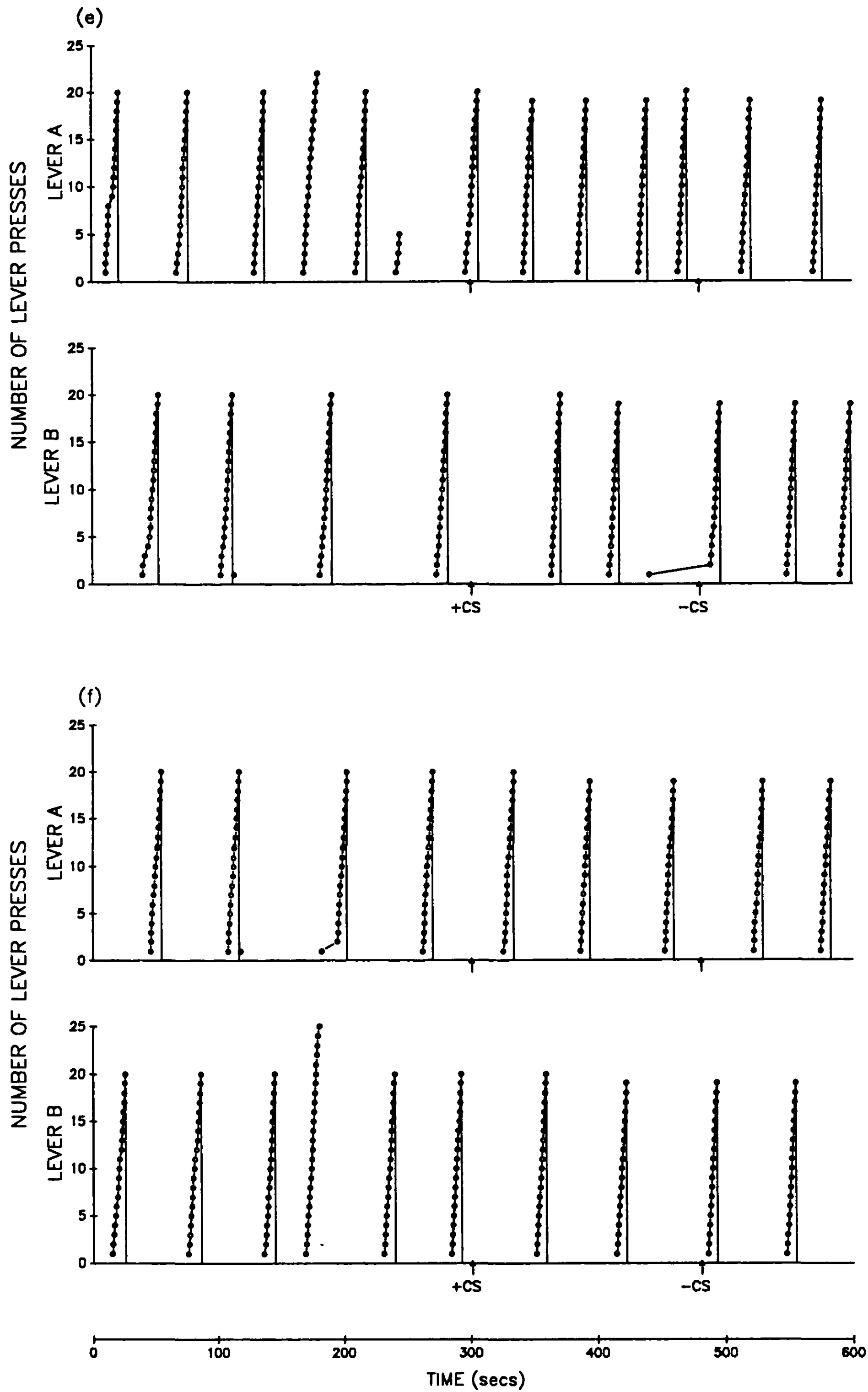


Fig 6.2 contd. (e) effect of a treatment with the food vehicle alone on the response to the conditioned tone stimulus which was not paired with shock on the previous evening, (f) response to the conditioned tone stimulus in the absence of a tone/shock pairing conditioning on the previous evening.

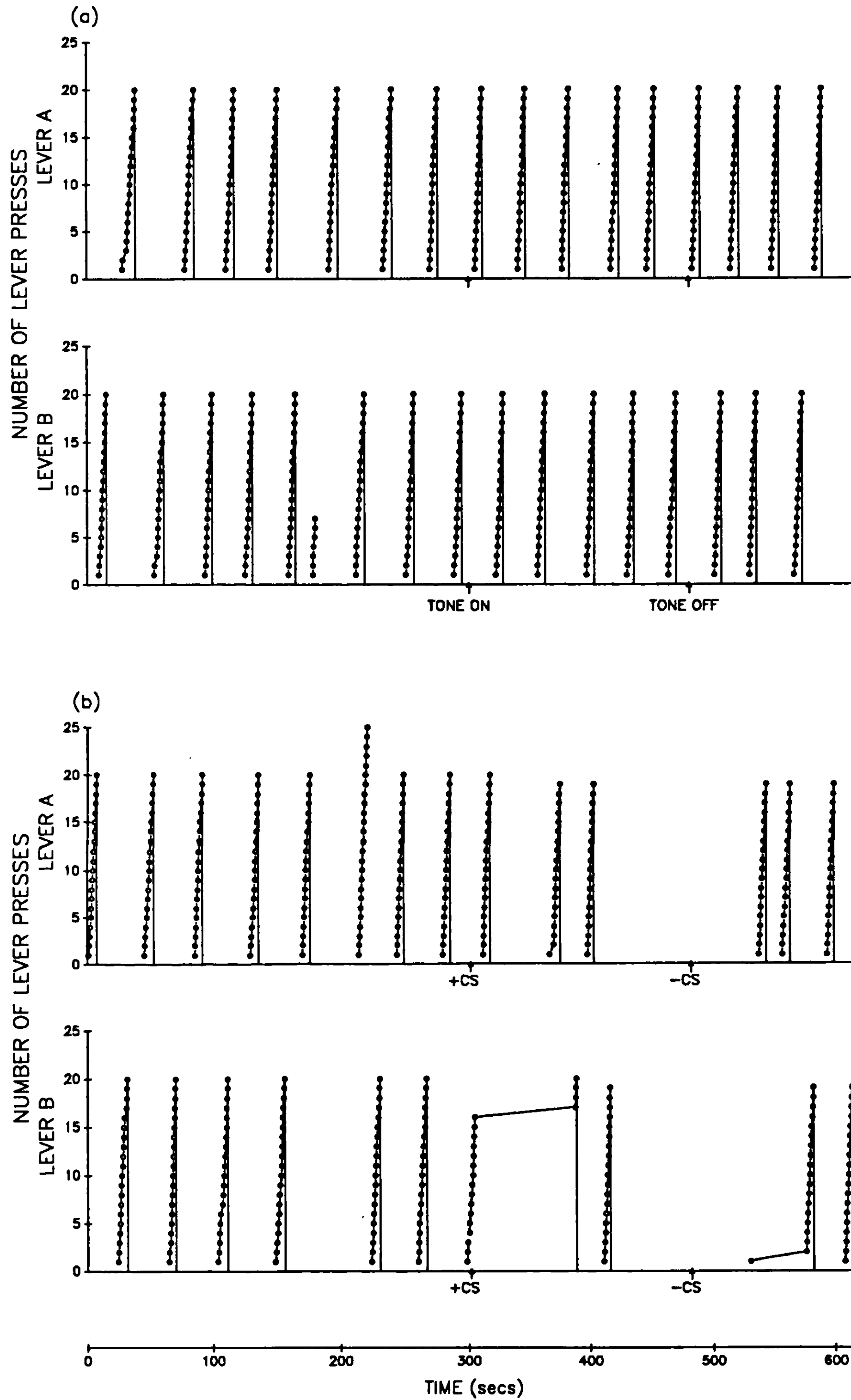


Fig 6.3 Cumulative records of the discriminatory response of pig C5 during the acquisition of a conditioned emotional state induced by a presentation of an aversively conditioned tone stimulus for a duration of 180 secs starting 300 secs after a response to a saline pretreatment: (a) response to a neutral tone stimulus, (b) response to the conditioned tone stimulus following a second tone/shock pairing conditioning on the previous evening.

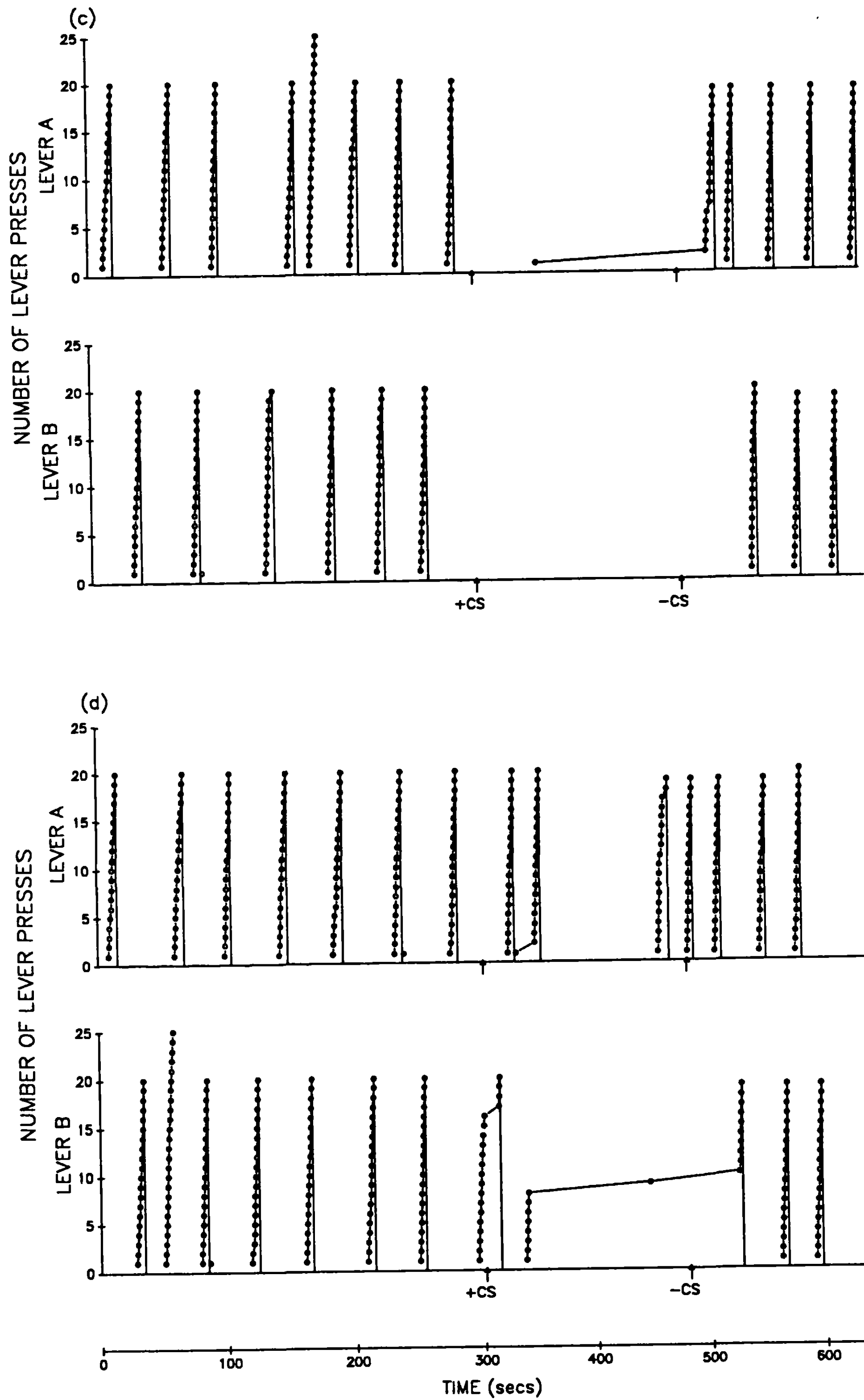


Fig 6.3 contd. (c) response to the conditioned tone stimulus in the absence of a tone/shock pairing conditioning on the previous evening, (d) response to the conditioned tone stimulus in the absence of a tone/shock pairing conditioning on the previous evening.

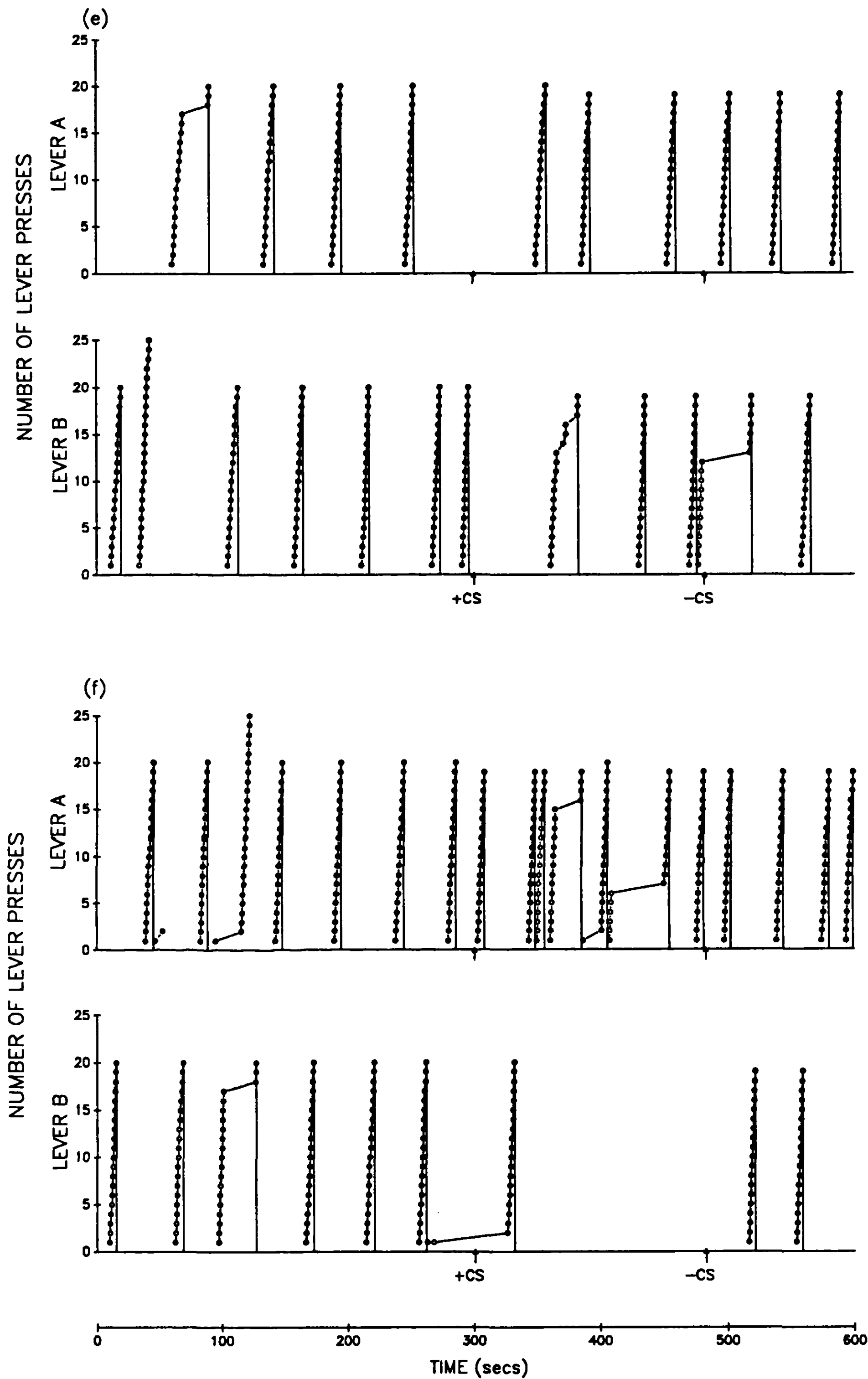


Fig 6.3 contd. (e) effect of a diazepam (0.5 mg/kg, p.o., 25 min) pretreatment on the response to the conditioned tone stimulus which was not paired with shock on the previous evening, (f) effect of a treatment with the food vehicle alone on the response to the conditioned tone stimulus which was not paired with shock on the previous evening.

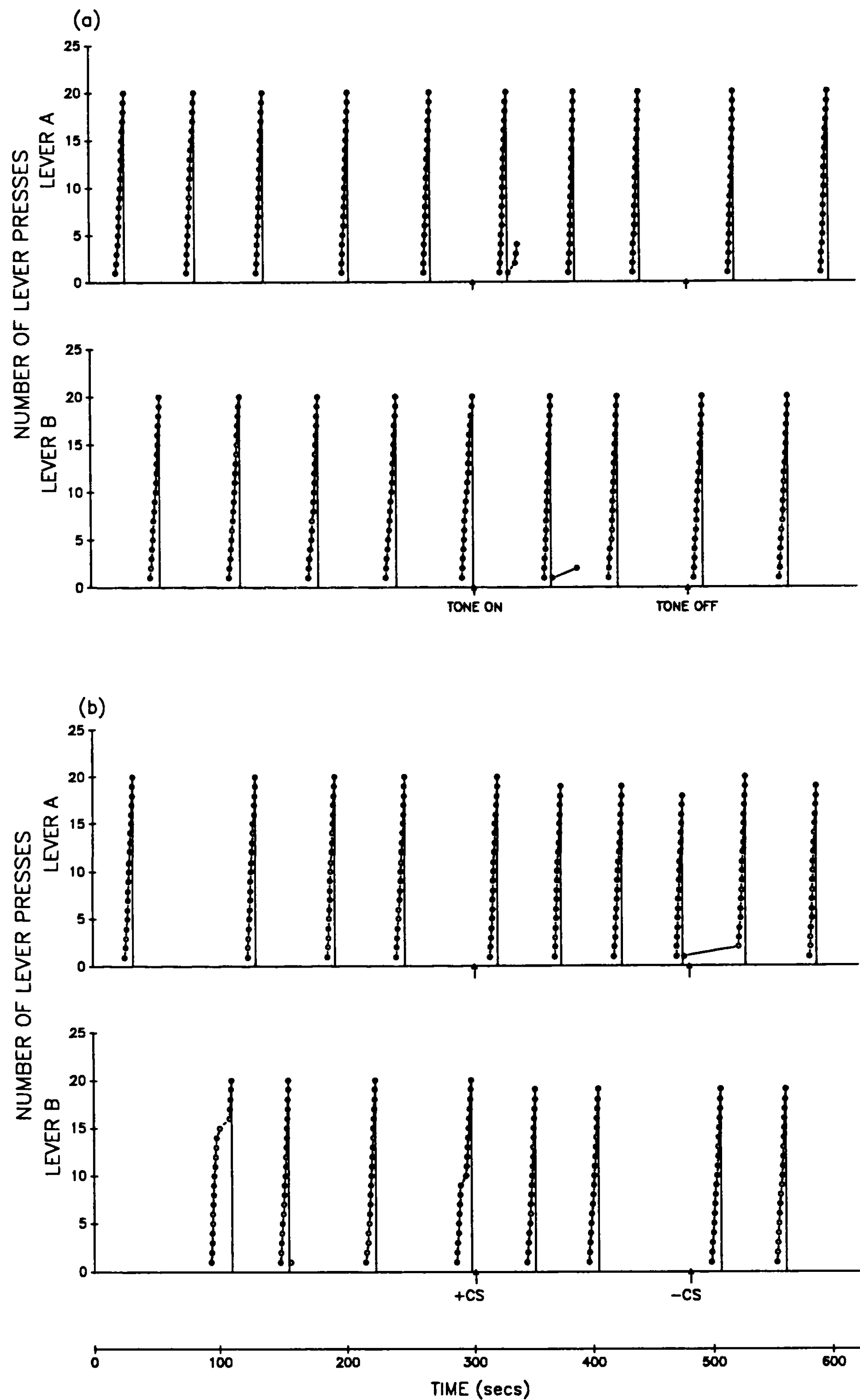


Fig 6.4 Cumulative records of the discriminatory response of pig C3 during the acquisition of a conditioned emotional state induced by a presentation of an aversively conditioned tone stimulus for a duration of 180 secs starting 300 secs after a response to a saline pretreatment: (a) response to a neutral tone stimulus, (b) response to the tone stimulus following a tone/shock pairing conditioning on the previous evening.

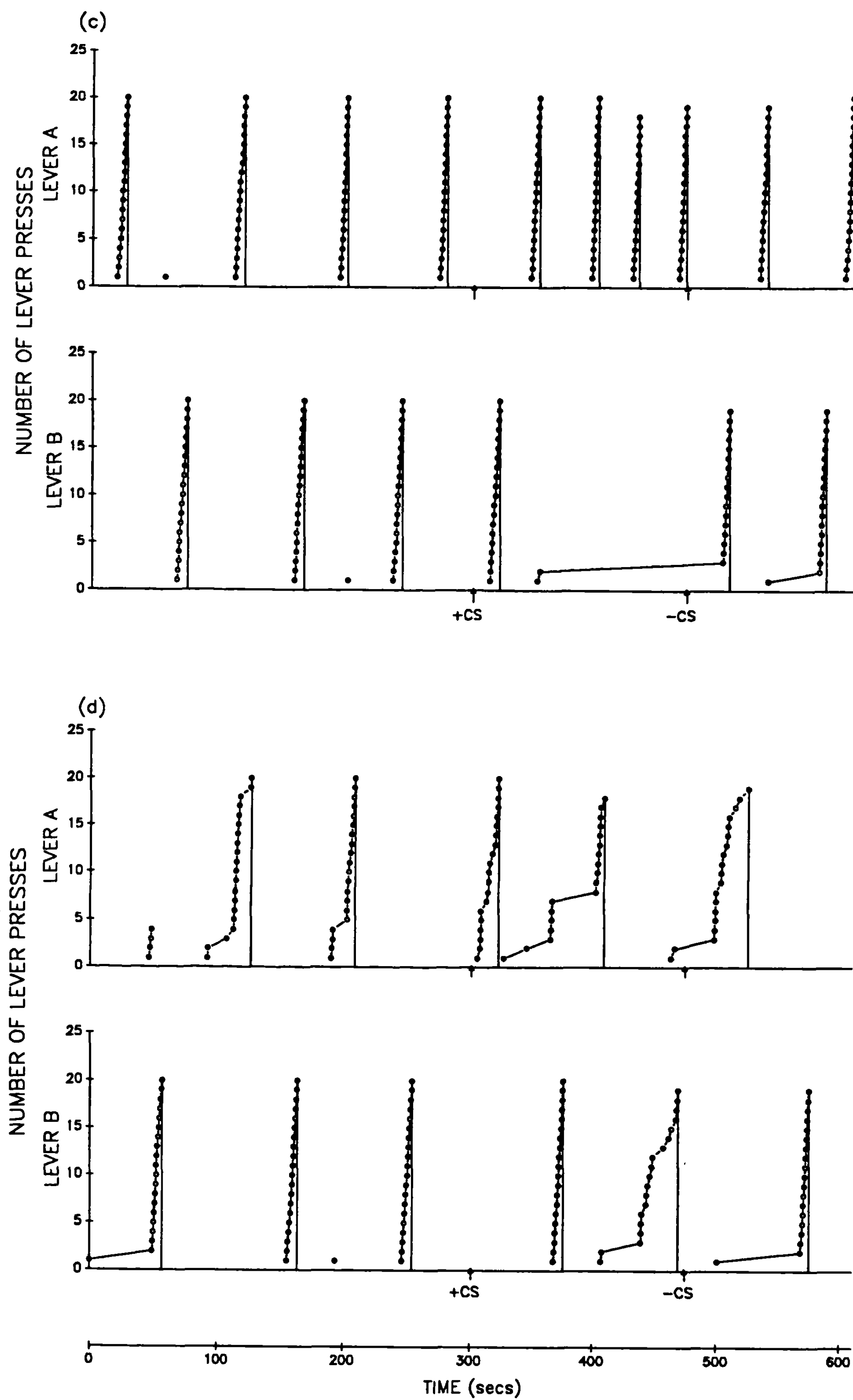


Fig 6.4 contd. (c) response to the conditioned tone stimulus following a second tone/shock pairing conditioning on the previous evening, (d) effect of a diazepam (0.5 mg/kg, p.o., 25 min) pretreatment on the response to the conditioned tone stimulus following a third tone/shock pairing conditioning on the previous evening.

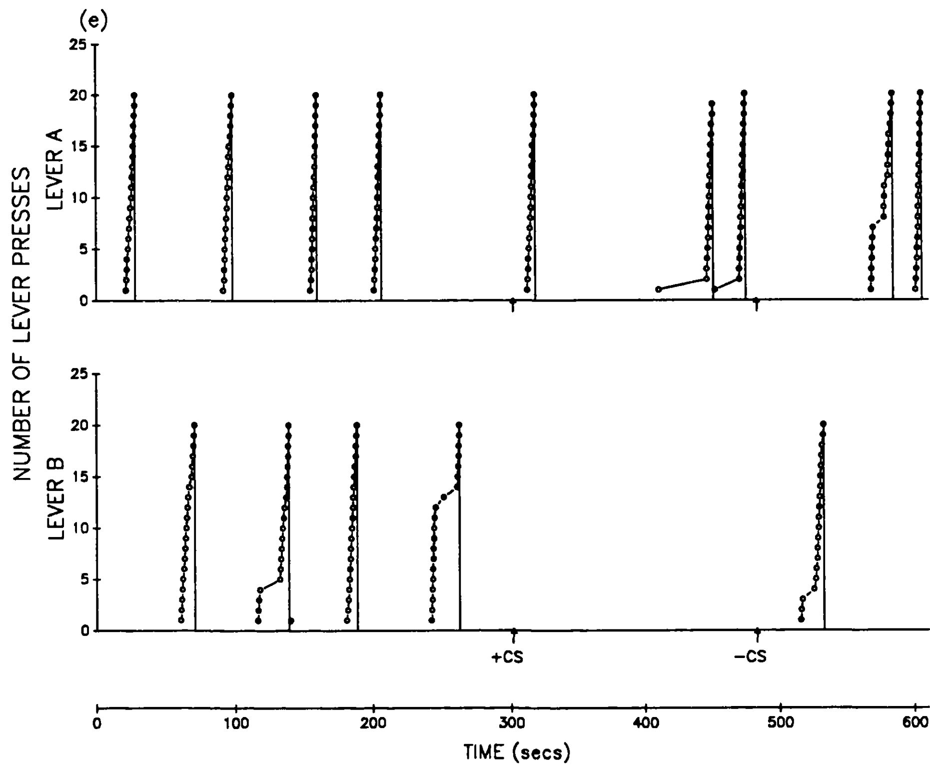


Fig 6.4 contd. (e) effect of a treatment with the food vehicle alone on the response to the conditioned tone stimulus following a fourth tone/shock pairing conditioning on the previous evening.

Effect of the CS tests on the maintenance of the discrimination criterion

The performance of pigs C1 and C5 in some of the saline training sessions during the period of tests with the CS did not satisfy the set discrimination criterion (see Chapter 2, Section 2.3.d, Procedure 2B). However, despite this finding, the study of the effect of the CS on the discriminatory response of these animals was continued without attempting to retrain the animals to satisfy the criterion. This approach was followed for two reasons. Firstly, the study of the CES was conducted on a continuous day-to-day basis, whereby the response to the CS was monitored each day either during the acquisition or extinction of the CES (see above). A disruption of this study for a several day retraining period would have necessitated the neutralisation of the tone stimulus followed by further conditioning trials of tone/shock pairings. As described above, repeated administration of shock to pig C3 resulted in aberrant behaviour in subsequent training sessions and therefore attempts were made to avoid the occurrence of this situation with pigs C1 and C5. Secondly, some of the incorrect selections made by these animals during the saline training sessions may have represented a response to some aversive aspect of the Skinner box since the tone/shock pairings were administered in this apparatus with the two levers removed. The performance of pig C3 in saline training sessions during the period of CS tests was within the discrimination criterion but the tone/shock pairings were administered to this animal in a temporary pen constructed in the conditioning room. Attempts to use this apparatus to condition the tone stimulus in pigs C1 and C5 were unsuccessful. Although these animals showed escape behaviour on repeated conditioning trials in the temporary pen, subsequent presentation of the CS during saline sessions in the Skinner box had no effect on the alternation of lever selection response. Since a disruption of operant response was observed in pig C3 at the end of the period of CS tests, it is clear that some aspect of the conditioning room also served as a pseudo-CS for this pig. Lane et al. (1982) have shown that administration of electric shocks to rats in a modified operant chamber induced neurochemical changes in these animals during subsequent operant performance in the apparatus. Since some of these changes were also

produced in animals presented with a conditioned tone stimulus, the authors conclude that the operant chamber may have represented a pseudo-CS to the shock-only animals. This explanation might also account for the present observations of incorrect selections of the drug designated lever during saline sessions in the absence of a CS presentation.

The incorrect lever selections made by pig C1 in the saline sessions during the period of CS tests are shown in Fig. 6.5. Saline sessions out of criterion occurred after the first tone/shock pairing and also after the second tone/shock pairing. The finding that all of the incorrect selections were on the drug designated lever supports the above suggestions that such selections may represent a response to a pseudo-CS. The incorrect lever selections made by pig C5 in saline sessions during the period of CS tests are shown in Fig. 6.6. Saline sessions out of criterion occurred after the third tone/shock pairing and before each subsequent CS test until the second last test before extinction of the CES was observed (see Table 6.1). As for pig C1, there were more incorrect selections on the drug designated lever than on the alternate lever.

It is clear from Fig. 6.5 and Fig. 6.6 that the incorrect selections made by these pigs did not occur at a set time during the 10 min sessions. For each animal, the total number of incorrect selections were pooled and the distribution of these selections in each 150 sec block of the 10 min training sessions was analysed by the Chi-square test. This analysis is shown in Table 6.2. There was no significant difference between the observed distribution of incorrect selections and that expected from a random distribution over the 10 min test period. This result eliminates the possibility that the selections of the drug designated lever by these animals during the 3 min CS presentation was due to a non-specific aversion to the test apparatus rather than a specific response to the conditioned tone. This is further supported by the finding that a stable baseline of alternation responding was observed in each animal before presentation of the CS.

Table 6.2 The distribution of incorrect lever selections made by pigs C1 and C5 over the four 150 sec time periods of 10 min saline training sessions (pig C1, n=3; pig C5, n=13) conducted on the same day as tests with the conditioned stimulus. The distribution expected if these selections occurred at random is shown in parentheses. P > 0.05 expected **v** observed distribution; Chi-Square test.

Pig C1				
Incorrect Lever Selections	Test time (secs)			
	0-150	150-300	300-450	450-600
Lever A	1 (1.75)	4 (1.75)	2 (1.75)	0 (1.75)
Lever B	0 (0)	0 (0)	0 (0)	0 (0)
Pig C5				
Incorrect Lever Selections	Test time (secs)			
	0-150	150-300	300-450	450-600
Lever A	5 (3.5)	4 (3.5)	6 (3.5)	1 (3.5)
Lever B	2 (3.5)	4 (3.5)	3 (3.5)	3 (3.5)

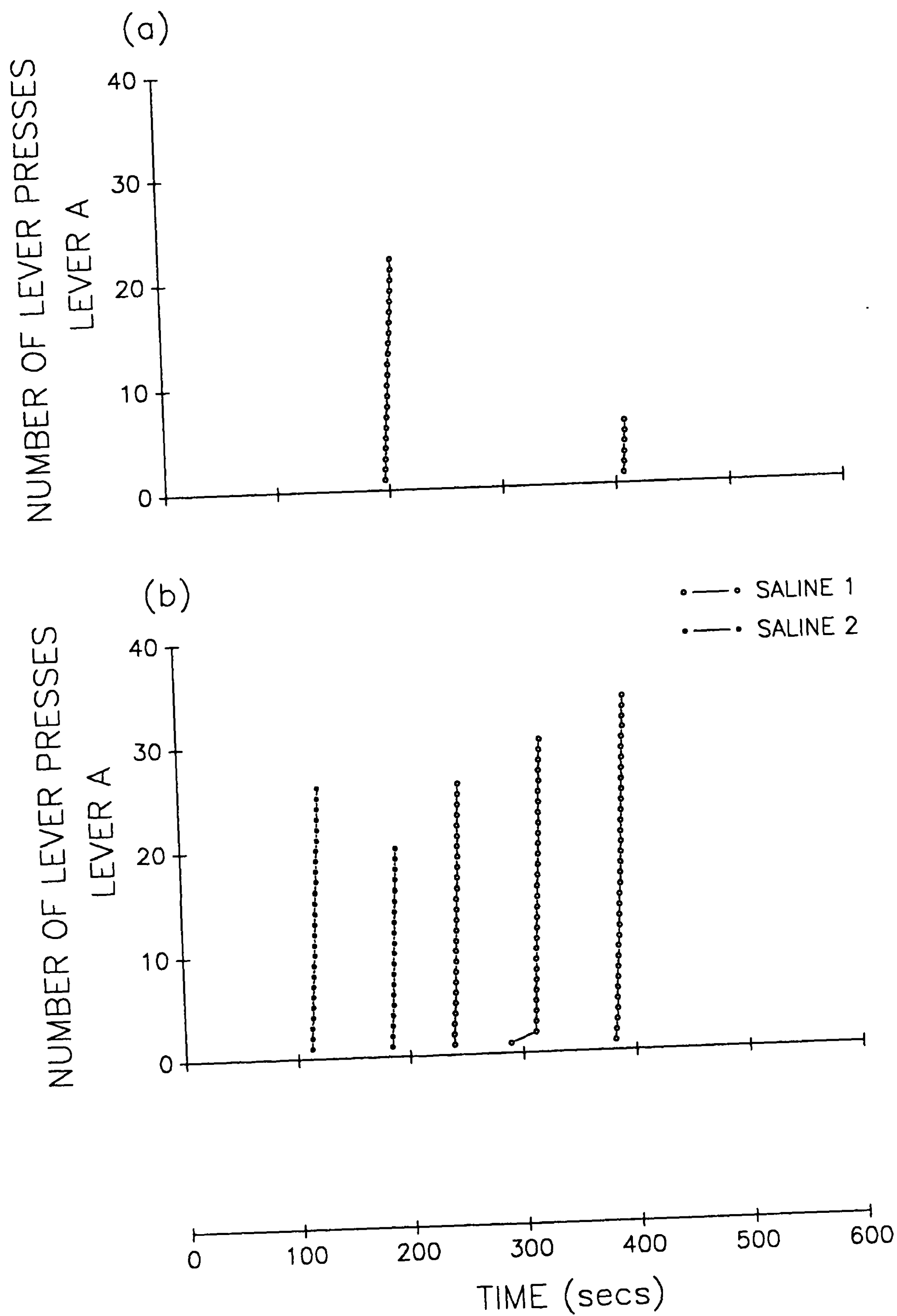


Fig. 6.5 Cumulative records of the response of pig C1 on the incorrect lever following a saline pretreatment: (a) during a training session following the first tone/shock pairing conditioning, (b) during training sessions following the second tone/shock pairing conditioning.

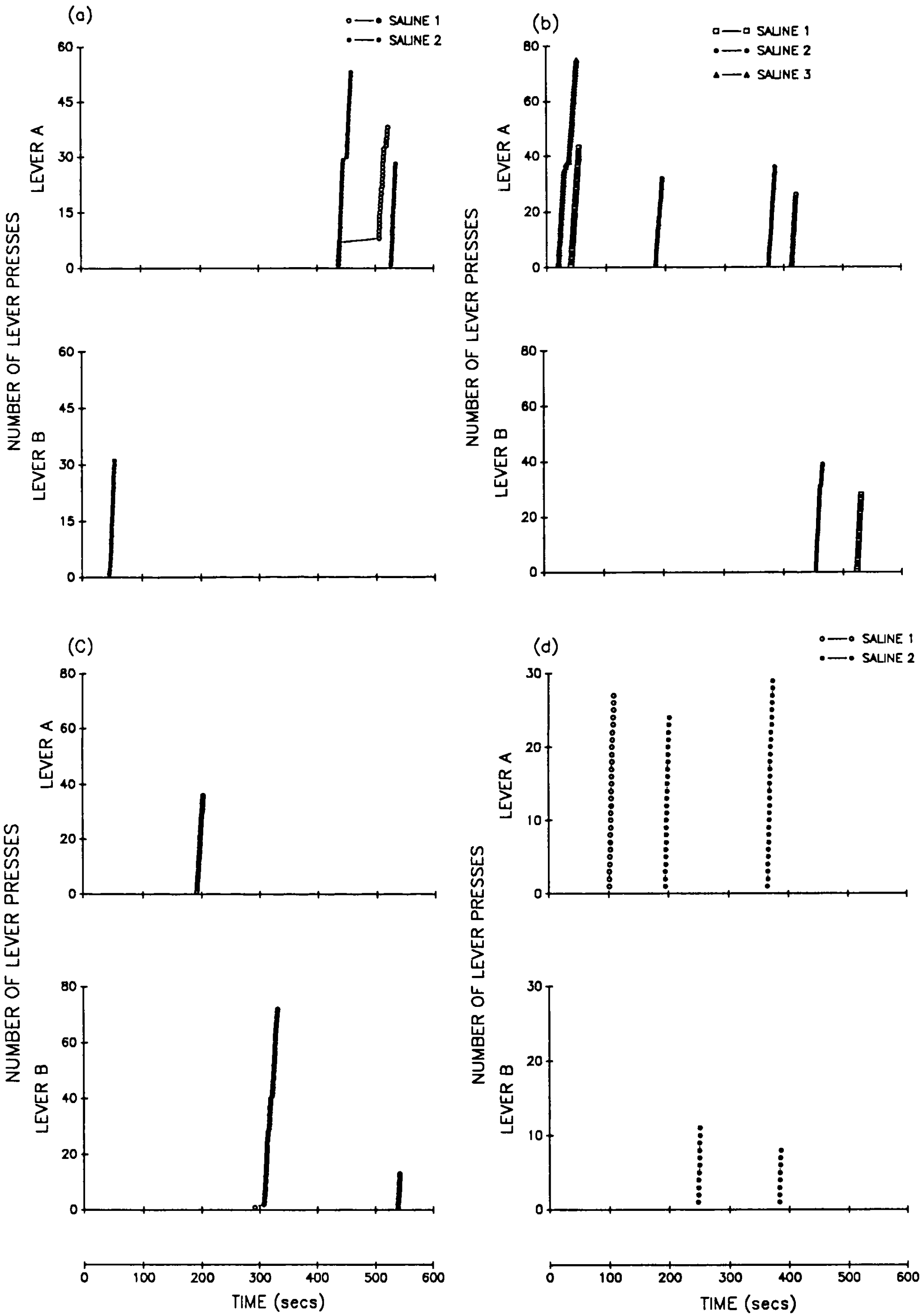


Fig. 6.6 Cumulative records of the response of pig C5 on the incorrect lever during training sessions following a saline pretreatment: (a) following the third tone/shock pairing conditioning, (b) before the fourth test with the conditioned stimulus, (c) before the fifth test with the conditioned stimulus, (d) before the sixth test with the conditioned stimulus.

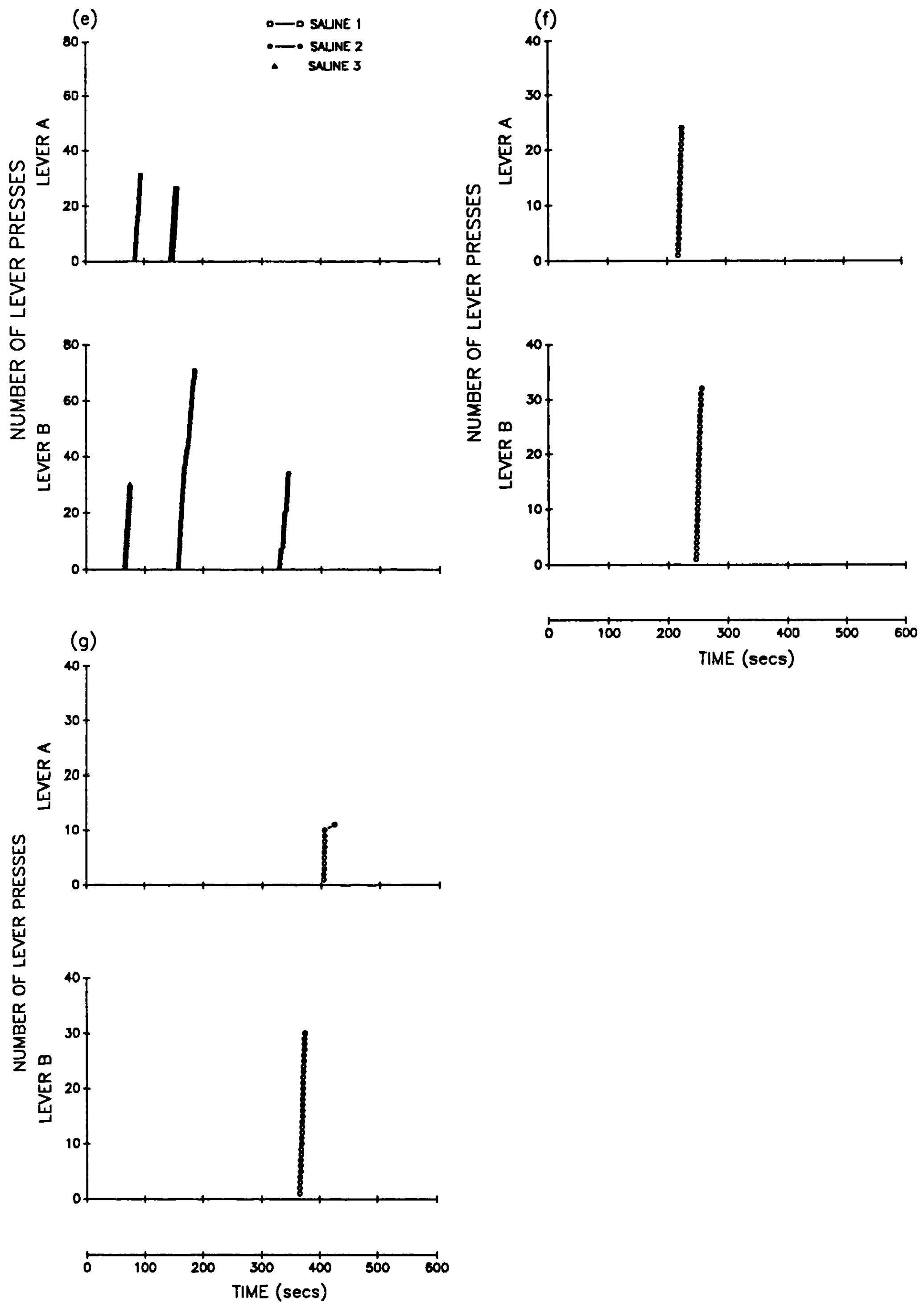


Fig. 6.6 contd. Cumulative records of the response of pig C5 on the incorrect lever during training sessions following a saline pretreatment:(e) before the seventh test with the conditioned stimulus, (f) before the eighth test with the conditioned stimulus, (g) before the ninth test with the conditioned stimulus.

Additive effect of a subthreshold dose of PTZ and a CES undergoing extinction

The above results show that the state induced by a conditioned tone stimulus generalises to the PTZ cue. Since the CS is not known to induce any proconvulsant properties, this provides firm evidence that the PTZ cue corresponds to a state of anxiety. Hence, the diazepam induced antagonism of the CES represents an anxiolytic action of this drug. Since the CES generalised to the PTZ injected state, it might be expected that both of these psychological states arise through a common mechanism. This possibility was investigated by examining the effect of a combination of a non-discriminatory dose of PTZ and a CES undergoing extinction, on the discriminatory response of two animals.

Separate presentation of the CS in extinction or a dose of 2 mg/kg PTZ, to pig C1 did not affect the alternation of lever selection response to a saline pretreatment (Fig. 6.7). Likewise, separate application of the CS in extinction or a dose of 1.875 mg/kg to pig C5 resulted in an alternation of lever selection response (Fig. 6.8). However, presentation of the CS to pig C1 after 5 minutes of an alternation response followed by an infusion of 2 mg/kg PTZ at 6 min, resulted in a change in response from alternation of lever selection to a selection of the drug designated lever alone (Fig. 6.9.a). On removal of the CS the animal continued to select both levers alternately. A simultaneous application of the CS and 1.875 mg/kg PTZ to pig C5 after 5 min of an alternation of lever selection response, resulted in two consecutive selections of the drug designated lever (Fig. 6.9.b). This was followed by an alternation of lever selection response for the remainder of the 10 min test session. The subsequent presentation of a combination of the CS and a saline infusion to these animals did not affect the alternation of lever selection response to a saline pretreatment, nor did a further test with the subthreshold dose of PTZ alone (results not shown). The response of pig C1 to the combination of the CS and the subthreshold dose of PTZ was of greater magnitude than that observed in pig C5 and this probably reflects the difference in the timing of the application of the two stimuli to these animals. The results from this

investigation indicate that a common mechanism is responsible for both PTZ induced and behavioural conditioned anxiety.

Previous studies utilising the CER paradigm have relied on the suppression of operant response as an index of anxiety (Estes & Skinner,1941; Lauener, 1963; Baldwin & Stephens, 1973; Lane et al., 1982a, b). However, results from the present study demonstrate that an anxiogenic state is evident when the response of the animals during the CS period is maintained. A generalisation to the PTZ cue was observed in pig C3 (Fig. 6.1.c) before a further conditioning of the tone stimulus produced a suppression of response during application of the CS (Fig. 6.1.d). Likewise, for pig C5 a generalisation to the PTZ cue was evident (Fig. 6.3.b) before the CS produced a total suppression of response (Fig. 6.3.c). Conventional operant studies of the CER interpret a recovery of response during the CS period as an extinction of the CER. Results from the present study show that the CS may induce anxiety when tone/shock pairings are discontinued (see Figs. 6.3.d and 6.3.f). These findings demonstrate the inadequacy of conditioned response suppression as an index of anxiety.

Baldwin & Stephens (1973) demonstrated that during the acquisition of a CER, pigs displayed behaviour indicative of conditioned fear such as urination, defecation and crouching in anticipation of the shock. Such behaviour has also been reported by others using this paradigm in rats (e.g. Lane et al.,1982a, b). However, in the present study the above behaviour did not occur in response to the CS during the saline operant sessions or during the feeding sessions where the tone was conditioned. During the periods of suppressed operant response, the animals either stood still facing the levers or else they turned around in the box to face the exit. During the tone conditioning trials the pigs sometimes displayed escape behaviour and they usually vocalised on shock administration. The discrepancies between the behavioural findings of this study and those reported by Baldwin & Stephens (1973) may reflect the modified procedure employed in the present investigation, whereby each presentation of the CS during a saline session represented an extinction session since the tone was not terminated by shock.

Also, the pigs used in the present study had a history of PTZ conditioning and it is possible, therefore, that repeated administration of this drug may have induced some adaptation to processes which govern the expression of such emotional behaviour.

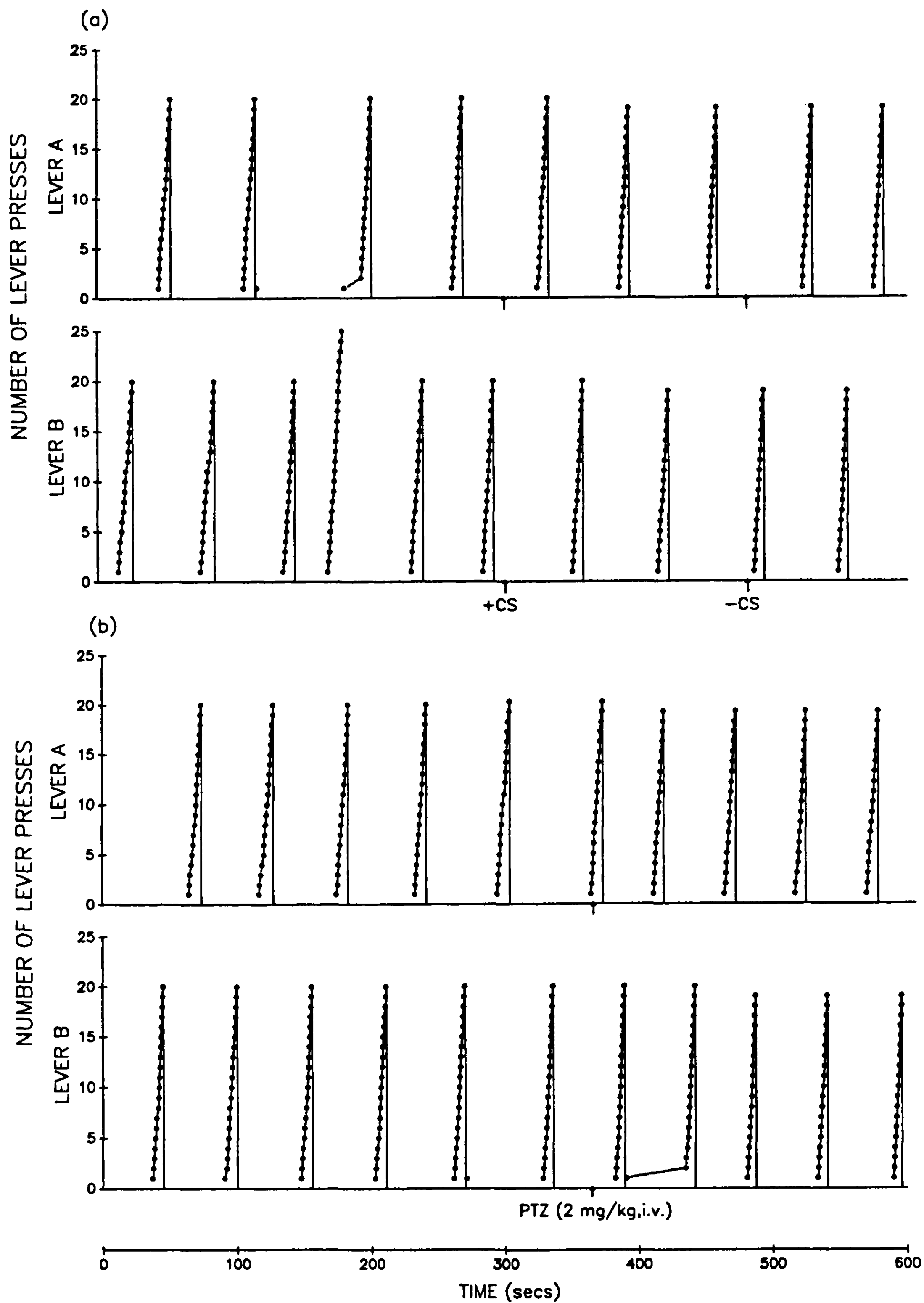


Fig. 6.7 Cumulative records of the discriminatory response of pig C1 to a conditioned emotional state undergoing extinction or a subthreshold dose of PTZ: (a) response to the conditioned stimulus which was presented for a duration of 180 secs starting 300 secs after a response to a saline pretreatment. The conditioned stimulus was similarly presented in three previous tests in the absence of tone/shock pairing conditioning, (b) response to an intravenous infusion of 2 mg/kg PTZ given 360 secs after a response to a saline pretreatment.

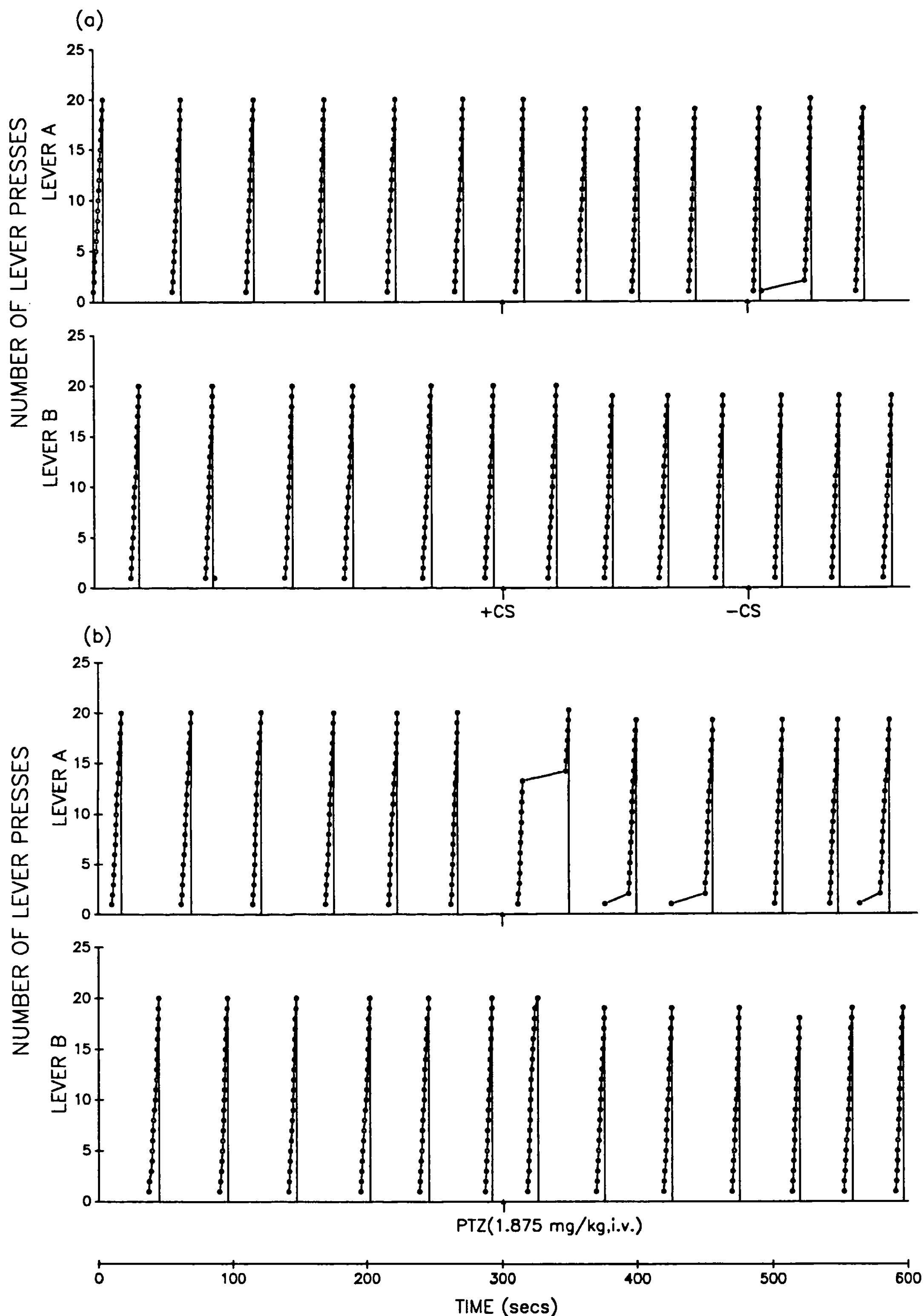


Fig. 6.8 Cumulative records of the discriminatory response of pig C5 to a conditioned emotional state undergoing extinction or a subthreshold dose of PTZ: (a) response to the conditioned stimulus which was presented for a duration of 180 secs starting 300 secs after a response to a saline pretreatment. The conditioned stimulus was similarly presented in eight previous tests in the absence of tone/shock pairing conditioning, (b) response to an intravenous infusion of 1.875 mg/kg PTZ given 300 secs after a response to a saline pretreatment.

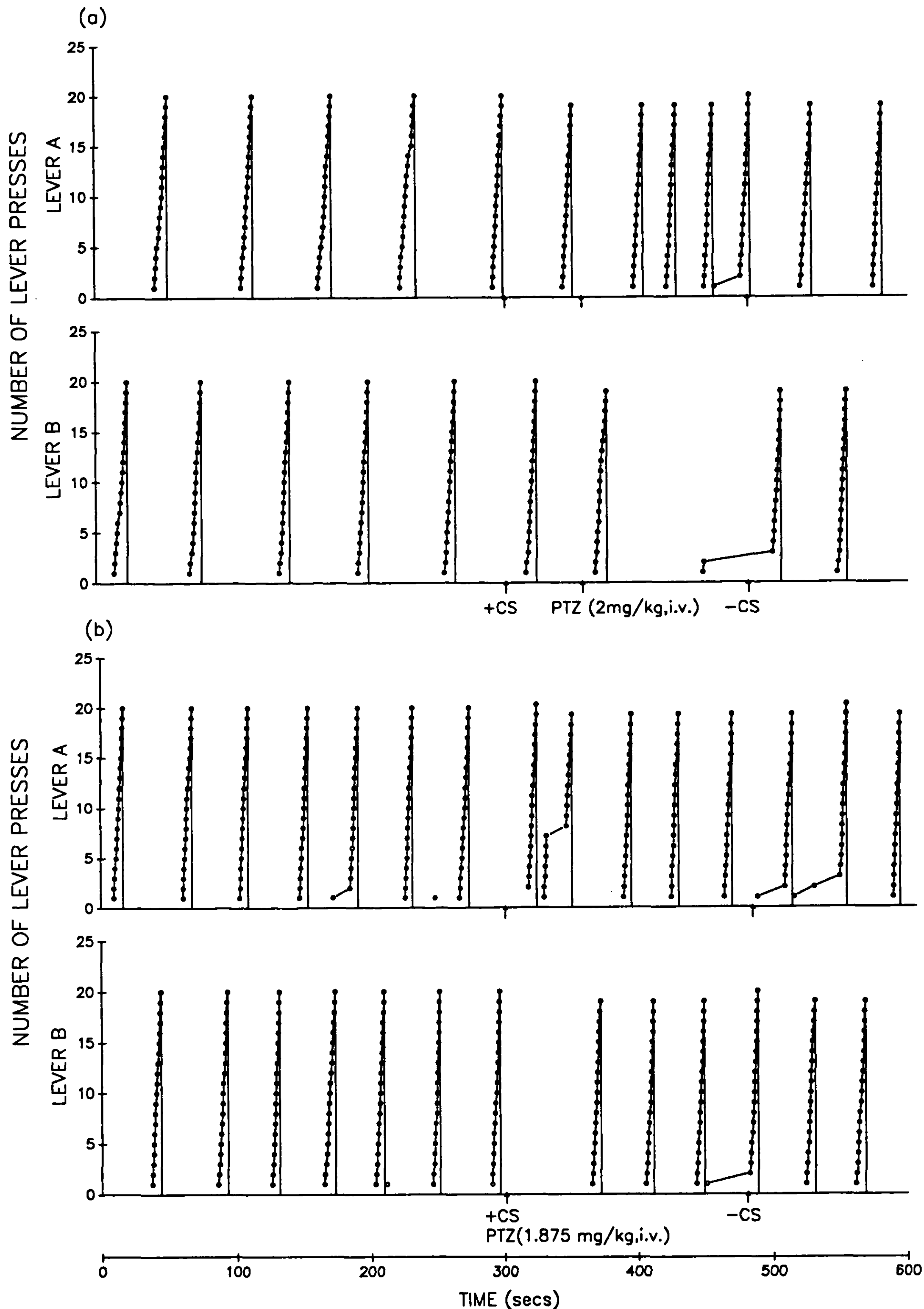


Fig. 6.9 Cumulative records of the discriminatory response of pigs to a combination of a conditioned emotional state undergoing extinction and a subthreshold dose of PTZ: (a) response of pig C1 to a presentation of a conditioned stimulus of duration 180 secs beginning 300 secs following the start of a saline session and an intravenous infusion of 2 mg/kg PTZ given 60 secs later, (b) response of pig C5 to a simultaneous presentation of a conditioned stimulus of duration 180 secs and an intravenous infusion of 1.875 mg/kg PTZ given 300 secs after a response to a saline pretreatment.

6.2.b A pharmacological evaluation of the PTZ stimulus

The behavioural evaluation of the PTZ cue described in the previous section showed that this cue corresponds to a state of anxiety. As discussed in Chapter 1 (Section 1.3.b), the pharmacological actions of PTZ are thought to be mediated through the picrotoxin site on the chloride channel of the $GABA_A$ receptor complex. Since other specific modulators of GABAergic function and anxiety are available, the pharmacological specificity of the PTZ cue can be investigated by examining the discriminatory responses of PTZ conditioned animals to such drugs. In the present study, FG7142 was tested for its ability to generalise to the PTZ cue, while diazepam was tested for its ability to antagonise this cue.

Although, the extensive pharmacological evaluation of the PTZ cue in rats, performed by Shearman & Lal (1980) indicated that the antagonism of the PTZ cue was specific to drugs with known anxiolytic actions, a more recent report has questioned this finding. Thus, Andrews et al. (1989) have demonstrated a positive correlation between the ability of a range of compounds to antagonise the PTZ discriminative stimulus and their ability to antagonise PTZ kindled seizures in rats. Of these compounds, the anticonvulsant ethosuximide has no known anxiolytic properties and indeed, anxiety is a reported side effect of this drug, particularly in patients with a prior history of psychiatric disturbance (Rall & Schleifer, 1985). In view of these conflicting reports, the effect of an anticonvulsant dose of ethosuximide on the PTZ cue in pigs was examined.

Effect of FG7142 on the PTZ cue

1. Pigs pharmacologically conditioned by procedure 2A (Chapter 2, Section 2.3.d)

Administration of FG7142 to pig 13 at doses of 5, 7.5 and 30 mg/kg, p.o. did not produce selection of the PTZ lever but rather selection of the saline lever (Fig 6.10a). The treatment to test time interval of 45 mins was chosen on

the basis of previous studies which demonstrated maximal effects of FG7142 within this time period, on anxiety in humans after oral administration (Dorow et al.,1983) and on proconvulsant activity in mice after an intraperitoneal injection (Little et al.,1984). The possibility that this drug might have produced a PTZ like stimulus in the pig after a shorter time interval was examined by testing pig 15, at 20 mins after treatment with 40 mg/kg FG7142. This test resulted in selection of the saline lever (Fig. 6.10b). However when the pig was retested 25 mins later (i.e. 45 mins after drug administration) the PTZ lever was selected (Fig. 6.10c). To ensure that this selection was not due to lack of reinforcement for selection of the saline lever in the first test, FG7142 (40 mg/kg, p.o.) was administered again on a separate day and when tested 45 mins after drug treatment the animal selected the PTZ lever (Fig 6.10d).

2. Pigs pharmacologically conditioned by procedure 2B (Chapter 2, Section 2.3.d)

Treatment of pig C1 with FG7142 (40 mg/kg, p.o; 45min) resulted in an alternation of lever selection response for the entire 10 min test period (result not shown). A similar result was obtained when this pig was retested 85 min after drug administration.

FG7142 (40 mg/kg, p.o.) was administered to pig C5 and the animal was then subjected to 5 test sessions, each of approximately 5 min duration, at 15 min intervals following drug administration. The results of these tests are shown in Fig. 6.11. This drug did not affect the alternation of lever selection response except during the test given 60 min after drug administration when there were two occurrences of two consecutive selections of the PTZ designated lever. The first consecutive pair of PTZ lever selections occurred at the start of the test and equalled a response time of 53.4 secs. The pig then selected the alternate lever and this was followed by another two consecutive selections of the PTZ designated lever. Pretreatment with the food vehicle alone did not affect the alternation of lever selection response when tests were conducted after the same time intervals as for the FG7142 tests (Fig. 6.12).

A study of FG7142 in humans has shown that the duration of anxiogenic effects of this drug varied from a period of 2 min to 2 hours (Dorow et al., 1983). It is possible, therefore, that the short duration of selection of the PTZ designated lever by pig C5 represents an anxiogenic response to FG7142. However, the study of Dorow et al. (1983) has revealed that such short lasting anxiogenic effects of an acute treatment with this drug in man were followed by further anxiety attacks of longer duration (> 20 min). In the present study, when pig C5 was tested 75 min after drug treatment, an alternation of lever selection response was observed. From these findings it is difficult to interpret the response of pig C5 on the drug designated lever at 60 min after FG7142 treatment, as an anxiogenic effect of this drug.

Although FG7142 was shown to generalise to the PTZ cue when pig 15 (conditioned by procedure 2A) was tested 45 min after drug administration, the study with pigs C1 and C5 did not repeat this finding. However, the assessment of the effect of FG7142 on the PTZ cue in pig 15 was based on a single lever selection and therefore, since the duration of this effect of FG7142 is unknown, such an effect may have resembled that observed on treatment of pig C5 with FG7142.

The failure to observe an indication of an anxiogenic action of FG7142 in pig C1 may be due to variations between pigs in their sensitivity to the anxiogenic effects of this drug. Such a variation in sensitivity to FG7142 appears to occur in humans and rats. This drug has been shown to cause anxiety in only 2 out of 12 human subjects (Dorow et al.,1983) and it produced a generalisation to the PTZ cue in only 75-77% of rats (Stephens et al., 1984; Vellucci et al., 1988). An added complexity to an interpretation of the present observations with FG7142 is provided by reports which reveal that the proconvulsant or anxiogenic actions of this drug do not show a linear dose response relation. An inverted U-shaped dose/response function has been demonstrated for the proconvulsant effects of FG7142 in mice (Little et al., 1984) and also for the generalisation of this drug to the PTZ cue in rats (Stephens et al., 1984; Vellucci et al., 1988).The ineffectiveness of higher doses of the drug in these paradigms is consistent with reports which demonstrated

an anticonvulsant action of FG7142 in mice in the dose range from 40-160 mg/kg, which appears not to be mediated through the benzodiazepine receptor, since it is insensitive to antagonism by Ro15-1788 (Petersen et al., 1983; Little et al., 1984). In the present study, it was not possible to examine the dose response function for the ability of FG7142 to generalise to the PTZ cue because of the limited availability of the drug and the large size of the animals.

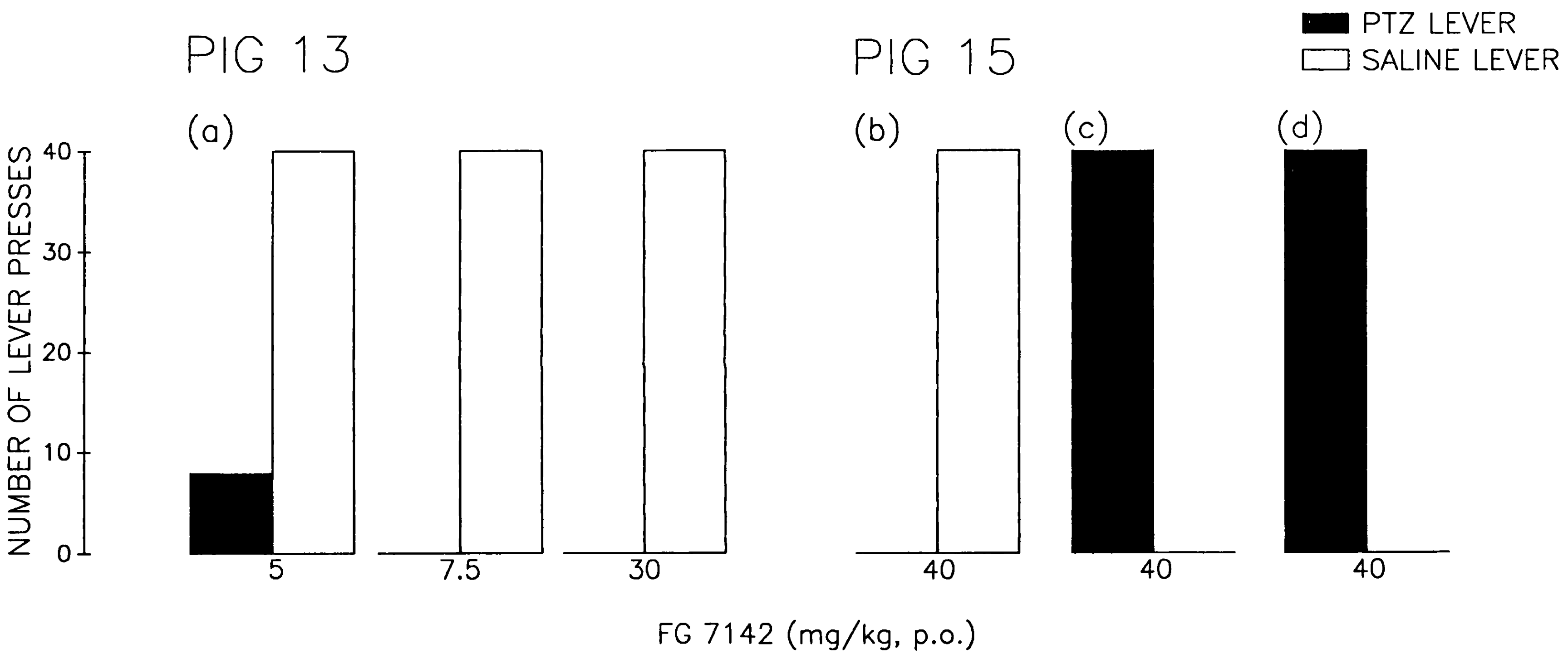
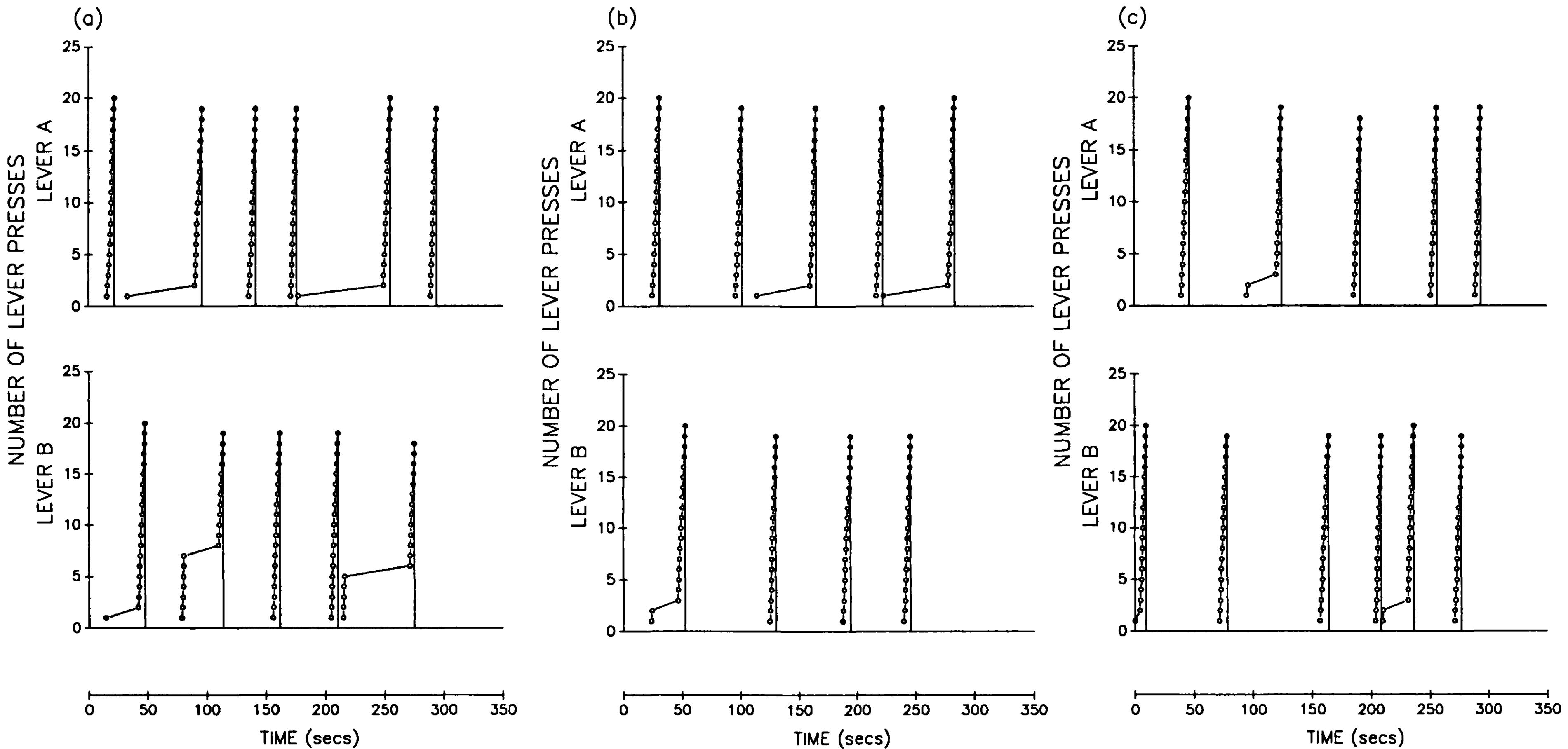


Fig. 6.10 Effect of FG7142 on the discriminatory response of pigs 13 and 15: (a) response of pig 13 on three occasions after the administration of FG7142 (5-30 mg/kg, p.o.; 45 min), (b) response of pig 15, 20 min after the administration of FG7142 (40 mg/kg,p.o.), (c) response of pig 15, 45 min after the administration of FG7142 (40 mg/kg, p.o.), (d) response of pig 15, 45 min after the administration of FG7142 (40 mg/kg, p.o.) on another day.


(a)
(b)
(c)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
TIME (secs)

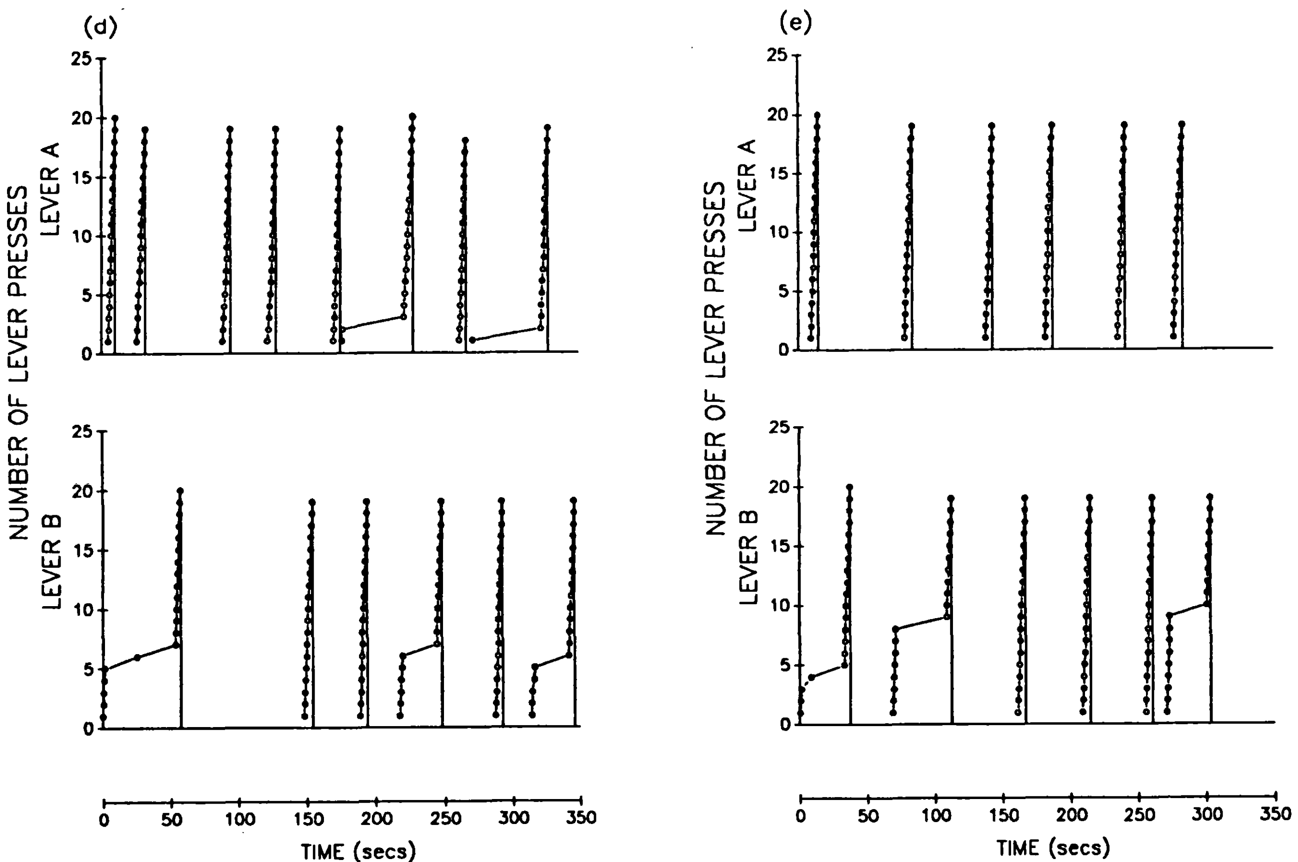
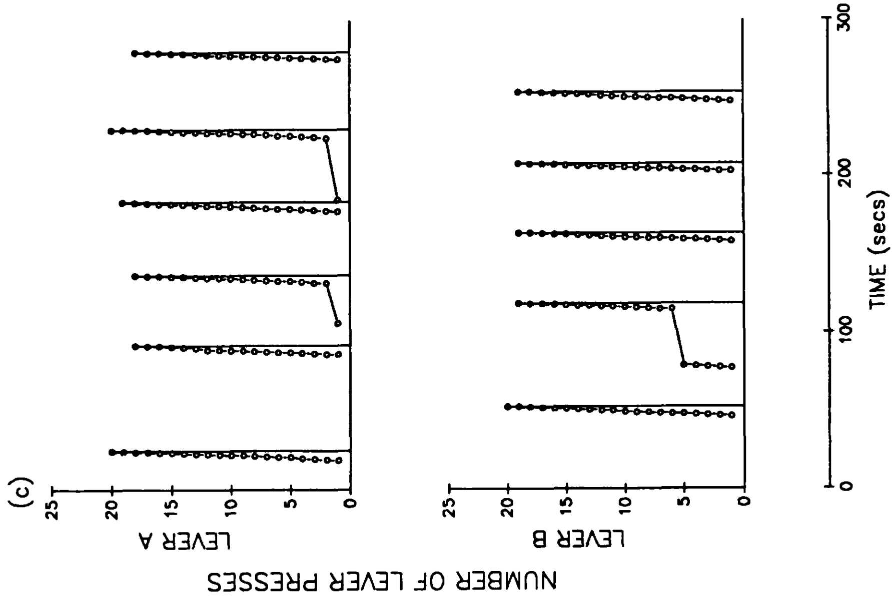
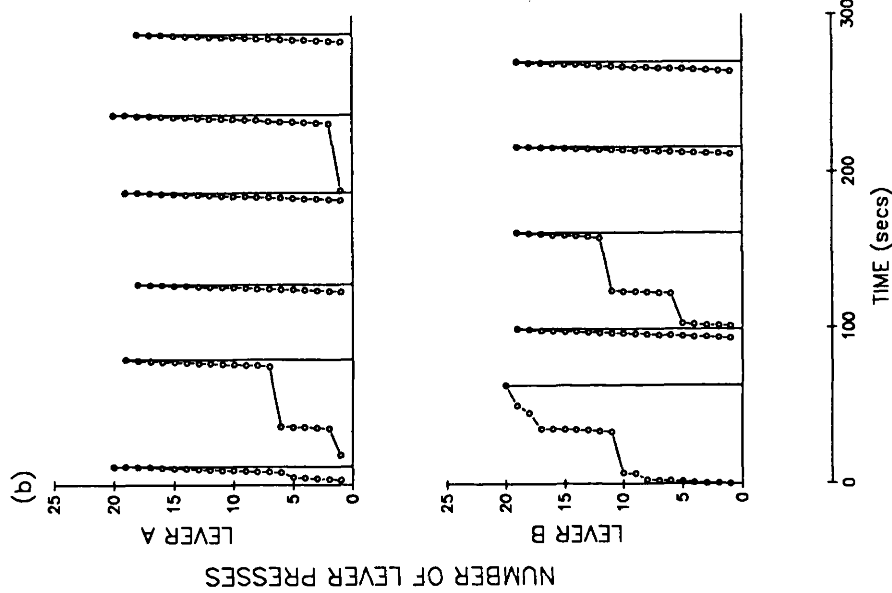
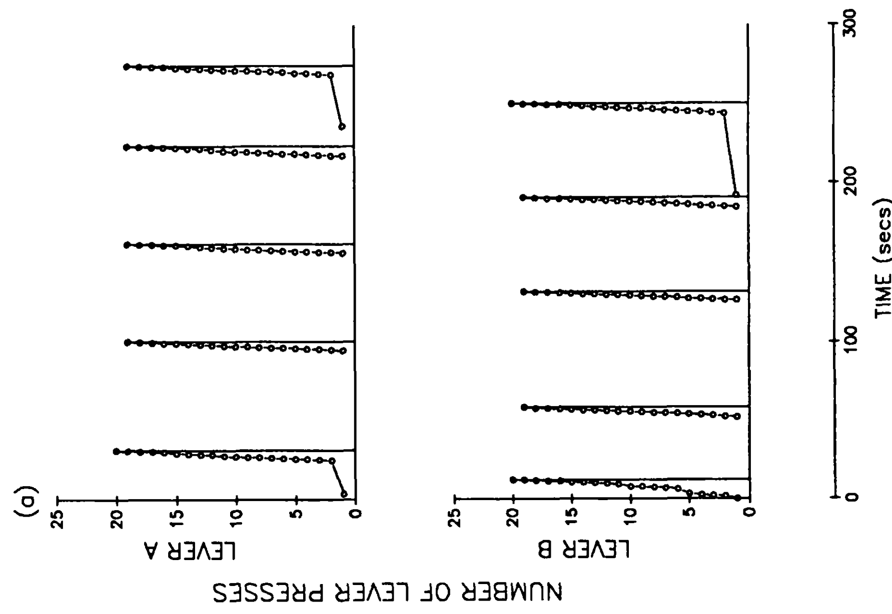


Fig. 6.11 Cumulative records of the discriminatory response of pig C5 to a pretreatment with FG7142 (40 mg/kg, p.o.) at the following times after drug treatment: (a) 15 min, (b) 30 min, (c) 45 min, (d) 60 min, (e) 75 min.


(c)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
TIME (secs)


(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
TIME (secs)


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
TIME (secs)

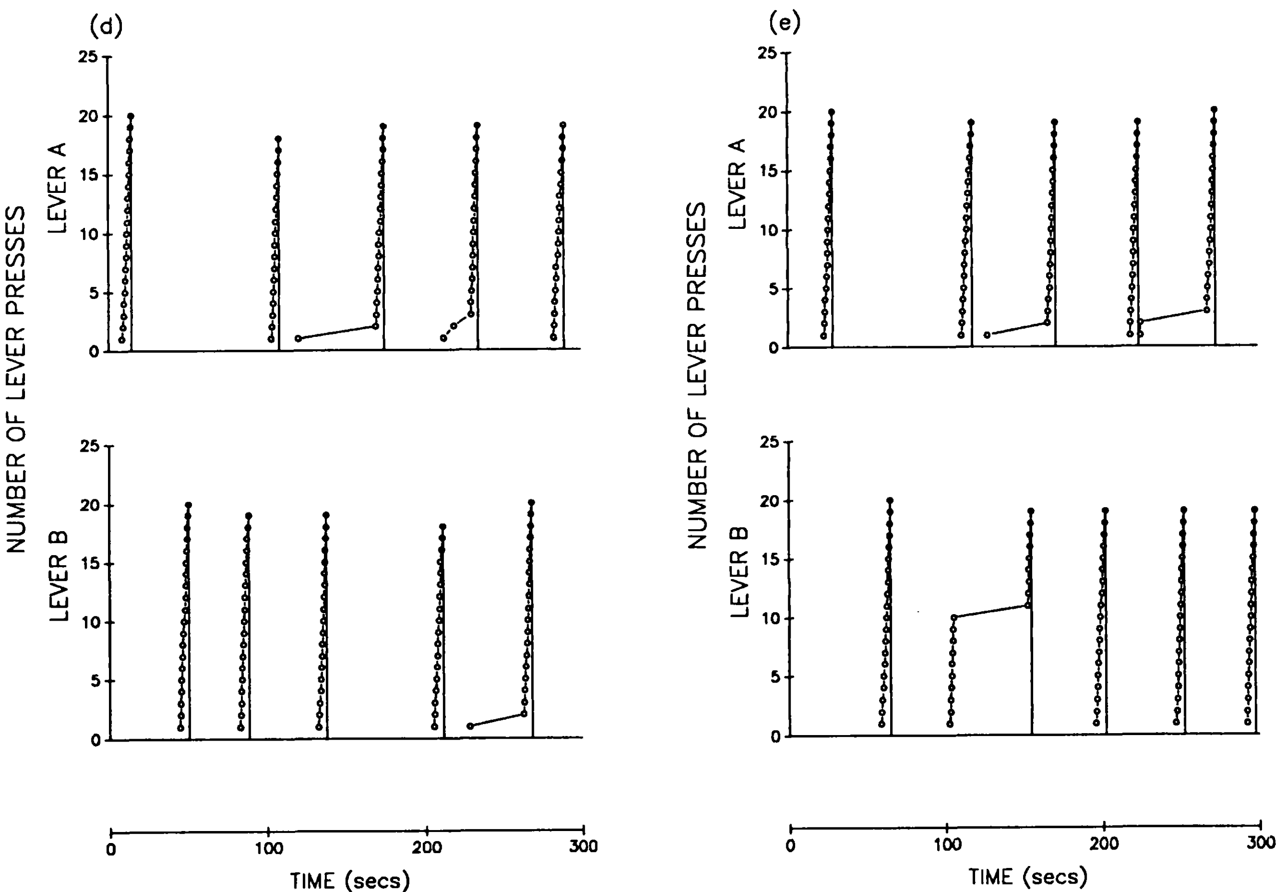


Fig. 6.12 Cumulative records of the discriminatory response of pig C5 to a treatment with the food vehicle alone at the following times after food administration: (a) 15 min, (b) 30 min, (C) 45 min, (d) 60 min, (e) 75 min.

Antagonism of the PTZ cue by diazepam

1. Pigs pharmacologically conditioned by procedure 2A (Chapter 2, Section 2.3.d)

The effect of pretreatment with diazepam (0.125-0.5 mg/kg, p.o.) on the response of pigs 13 and 15 to the training dose of PTZ (5 mg/kg, i.v.) is shown in Fig. 6.13. At each of the three diazepam doses tested, a complete antagonism of the PTZ stimulus was obtained in both pigs. Administration of the food vehicle alone did not affect the response of the animals to PTZ. The administration of Ro15-1788 (5 mg/kg, p.o.) alone did not affect the response of pigs 13 and 15 to PTZ but when administered prior to diazepam (0.5 mg/kg, p.o.) it blocked the ability of this drug to antagonise the PTZ stimulus (result not shown).

2. Pigs pharmacologically conditioned by procedure 2B (Chapter 2, Section 2.3.d)

Pretreatment of pigs C3 and C4 with diazepam (0.5 mg/kg, p.o.) resulted in an alternation of lever selection response to an infusion of the training dose of PTZ during a saline session (Figs. 6.14b and 6.15b). A previous presentation of the training dose of PTZ to these animals during a saline session produced a switch from an alternation of lever selection response to a response on the drug designated lever alone (Figs. 6.14a and 6.15a). The duration of this response was 122.1 secs for pig C3 and 60.1 secs for pig C4. Application of the training dose of PTZ to these pigs during a saline session, following a pretreatment with the food vehicle alone, also induced a change from an alternation of lever selection response to a response on the PTZ lever only (Figs. 6.14c and 6.15c). The duration of this effect was 157 secs for pig C3 and 52.1 secs for pig C4.

Following the CS tests described in the previous section, it was necessary to retrain pig C3 and during this training, the ratio of lever presses to food reward was increased to 40 as the response of the animal was more stable at this FR than at the previous FR 20.

The above results demonstrate a diazepam antagonism of the PTZ cue in pigs C3 and C4. Although the response of these animals to PTZ in the test sessions preceding and following the diazepam test did not differ significantly from the response to PTZ after diazepam treatment, it is clear from the records shown in Figs. 6.14 and 6.15 that an infusion of PTZ produced a consistent response on the PTZ lever which was antagonised by a diazepam pretreatment. The duration of the response of these animals to PTZ is in agreement with the time of action of this drug estimated from the training sessions conducted at this time with the same doses of the drug. From the training sessions, the mean response time on the PTZ lever was estimated at 98.78 secs for pig C3 and 59.6 secs for pig C4 (see Chapter 5, section 5.2.b, Table 5.4).

Fig 6.16b shows the effect of pretreatment with diazepam on the response of pig C5 to an infusion of the training dose of PTZ. Although, this animal started an alternation of lever selection response, a preference for the B lever developed during the test and in most cases two consecutive selections were made on this lever before the animal paused to consume the food reward. Injection of PTZ did not produce a selection of the drug designated lever or an alternation of lever selection response. Rather, the animal continued to select the B lever. A previous infusion of this dose of PTZ during a saline session produced a change in the response of the animal from alternation of lever selections to selections of the drug designated lever alone, for a period of 115.8 secs (Fig. 6.16a). The diazepam test was continued until 1050 secs had elapsed and at this time the response of the pig consisted almost entirely of selections of the B lever. When the animal was retested 30 min after the end of this test, both levers were selected alternately (Fig. 6.16c). The following day, the pig was pretreated with the food vehicle alone and presented with the training dose of PTZ during a saline session. This resulted in a switch from an alternation of lever selection response to a response on the drug designated lever alone (Fig. 6.16d). The duration of this response was 166.9 secs.

The ability of diazepam to antagonise the PTZ cue in this pig was further examined by subjecting the animal to another PTZ test following a diazepam pretreatment. On this occasion, the training dose of PTZ was

presented immediately before the start of the test session. The result of this test is shown in Fig. 6.17b. The animal responded to the PTZ injection by selecting both levers alternately. A previous infusion of PTZ during a saline session produced a selection of the drug designated lever for a period of 153.8 secs (Fig. 6.17a). Since a diazepam pretreatment resulted in an alternation of lever selection response to an injection of PTZ, this demonstrates the ability of diazepam to antagonise the PTZ cue. From Fig. 6.17b it is clear that as the diazepam test progressed, pig C5 again showed a preference for the B lever. After approximately 500 secs the animal was responding almost exclusively on this lever. The discriminatory response of this pig to diazepam was further examined in three subsequent tests of 5 min duration, each conducted at 30 min intervals following the diazepam test.The results of these tests are shown in Fig. 6.17c-e. The preference for the B lever persisted in the first two tests. When tested 141 min after diazepam administration, the animal responded by selecting both levers alternately (Fig. 6.17e). The day after these tests were conducted the pig was treated with the food vehicle alone and presented with the training dose of PTZ immediately before the start of a test session. In contrast to the result obtained after diazepam treatment, a preference for the PTZ designated lever was evident and a stable alternation of lever selection response did not occur until approximately 400 secs after the start of the test (Fig. 6.17f).

From the above results, it is clear that diazepam induced a preference in pig C5 for the B lever. Diazepam has been shown to enhance both the rate of response and the motivation for food in pigs performing an operant task for food reinforcement (Dantzer, 1978). While these findings may explain why pig C5 made consecutive selections of the B lever before consuming the food reward they do not explain the diazepam induced disruption of the alternation of lever selection response or the development of a preference for the B lever. Such a lever preference is unlikely to be due to the development of a spontaneous fixation on the B lever since the animal resumed a stable alternation of lever selection response in the absence of any negative consequences for continuous selection of this lever. An amnesic action of diazepam is unlikely to account for the present observations since the preference for the B lever occurred

consistently following the two treatments with this drug. It is possible that diazepam induced a cue which the animal could not compare with either the PTZ injected state or the undrugged state and therefore, a response unrelated to the typical response to either of these treatments was emitted.

The response of pig C5 on the PTZ designated lever was enhanced the day following the second diazepam treatment, whereby a stable alternation of lever selection response was not observed until approximately 400 secs after the PTZ injection. On the following day, an injection of the training dose of PTZ immediately before a test session resulted in a response on the drug designated lever for a period of 98.8 secs and this was followed by an alternation of lever selection response for the remainder of the test period (result not shown). The enhanced response of the animal to PTZ on the day following diazepam treatment may reflect an increased sensitivity to the anxiogenic action of PTZ, induced by withdrawal from an acute diazepam treatment. The state induced by withdrawal from chronic and acute benzodiazepine treatment in rats has been shown to generalise to the PTZ cue (Emmett-Oglesby et al., 1983; Michaelis et al., 1988; Barrett & Smith, 1988). In the present study such a generalisation was not observed but the anxiety state of the animal was evidently affected as revealed by the enhanced response to PTZ.

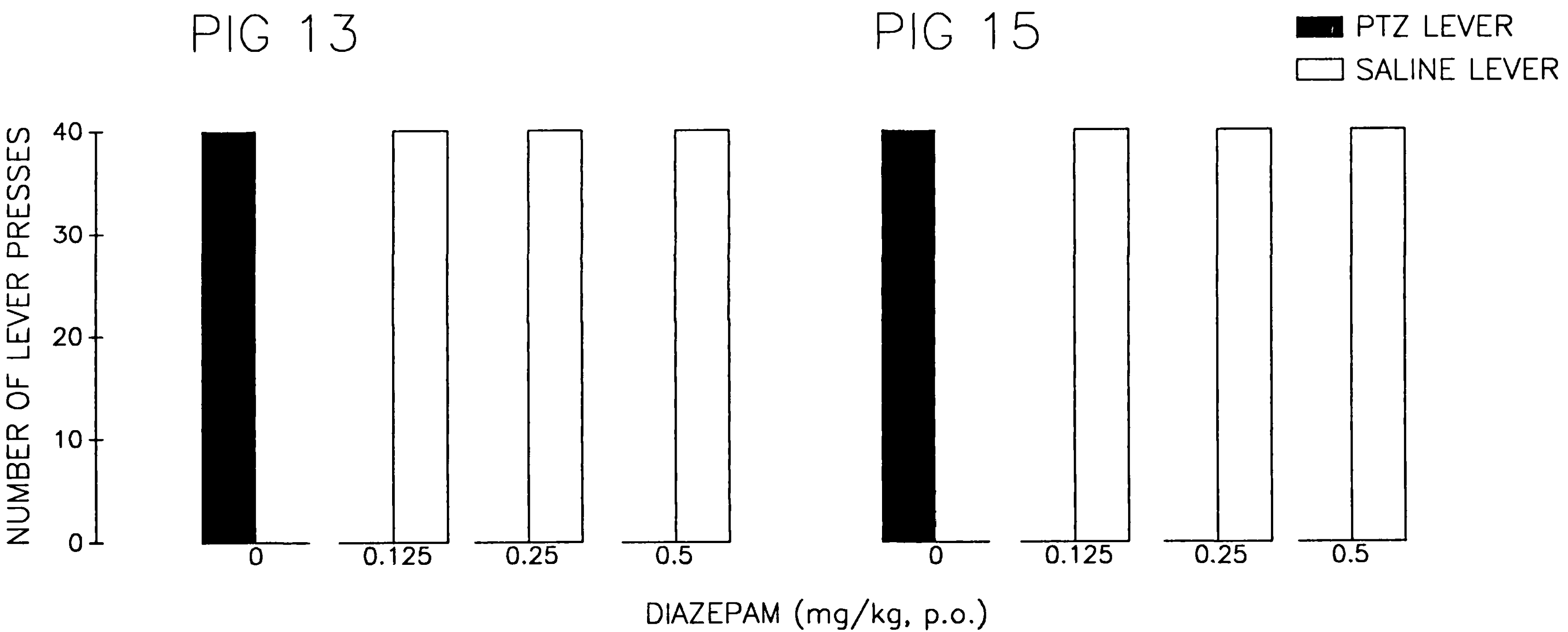
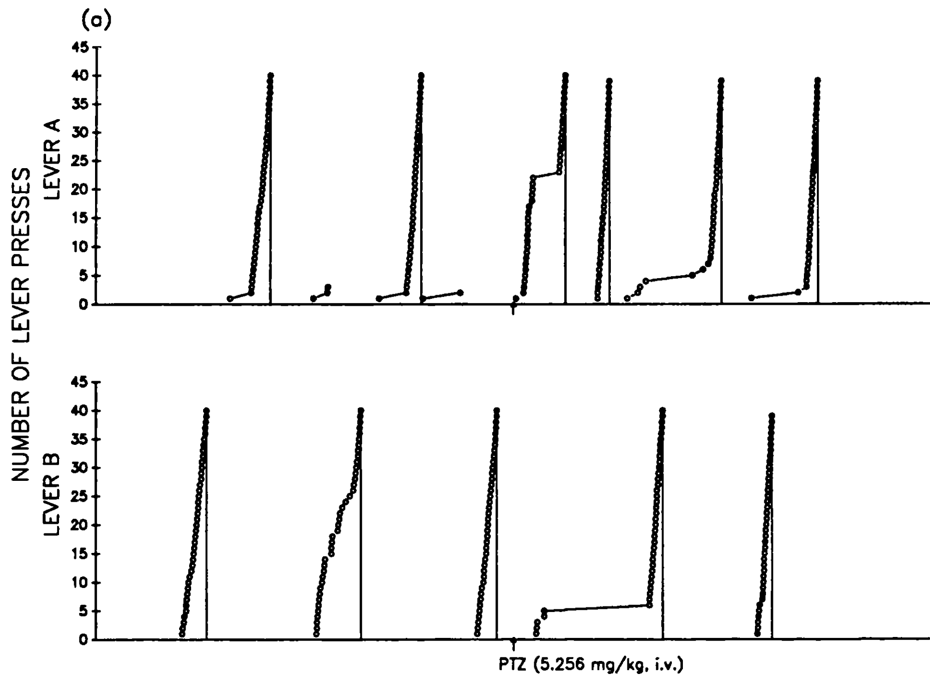
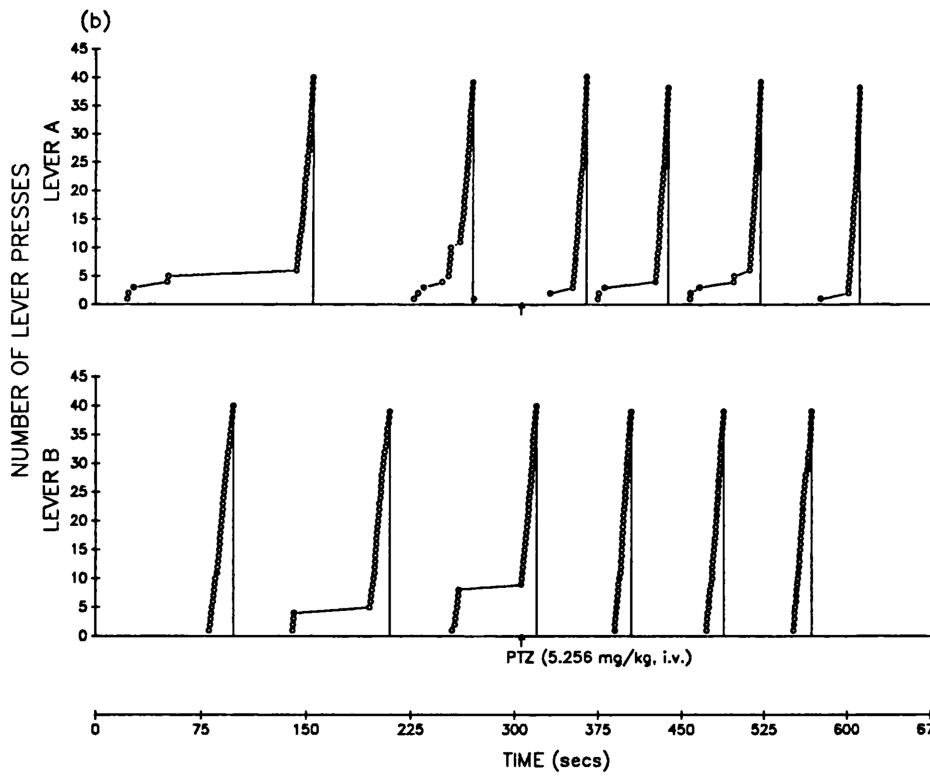


Fig. 6.13 Effect of a pretreatment with diazepam (0-0.5 mg/kg, p.o.; 30 min) on the discriminatory response of pigs 13 and 15 to the training dose of PTZ (5 mg/kg, i.v.).


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
45
40
35
30
25
20
15
10
5
0
PTZ (5.256 mg/kg, i.v.)


(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
45
40
35
30
25
20
15
10
5
0
PTZ (5.256 mg/kg, i.v.)
0 75 150 225 300 375 450 525 600 675
TIME (secs)

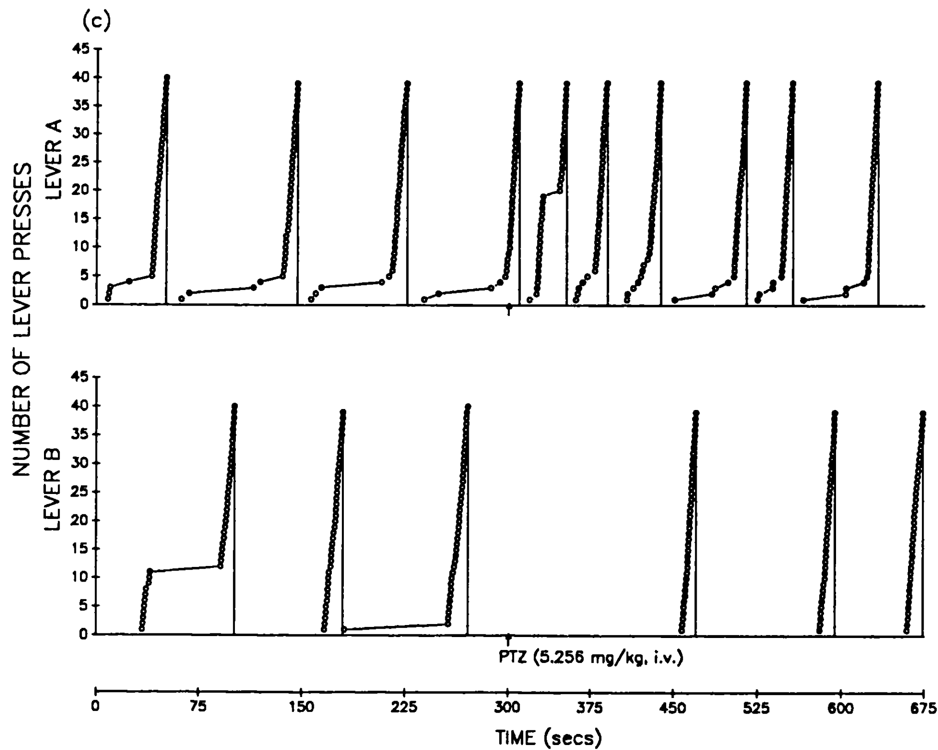
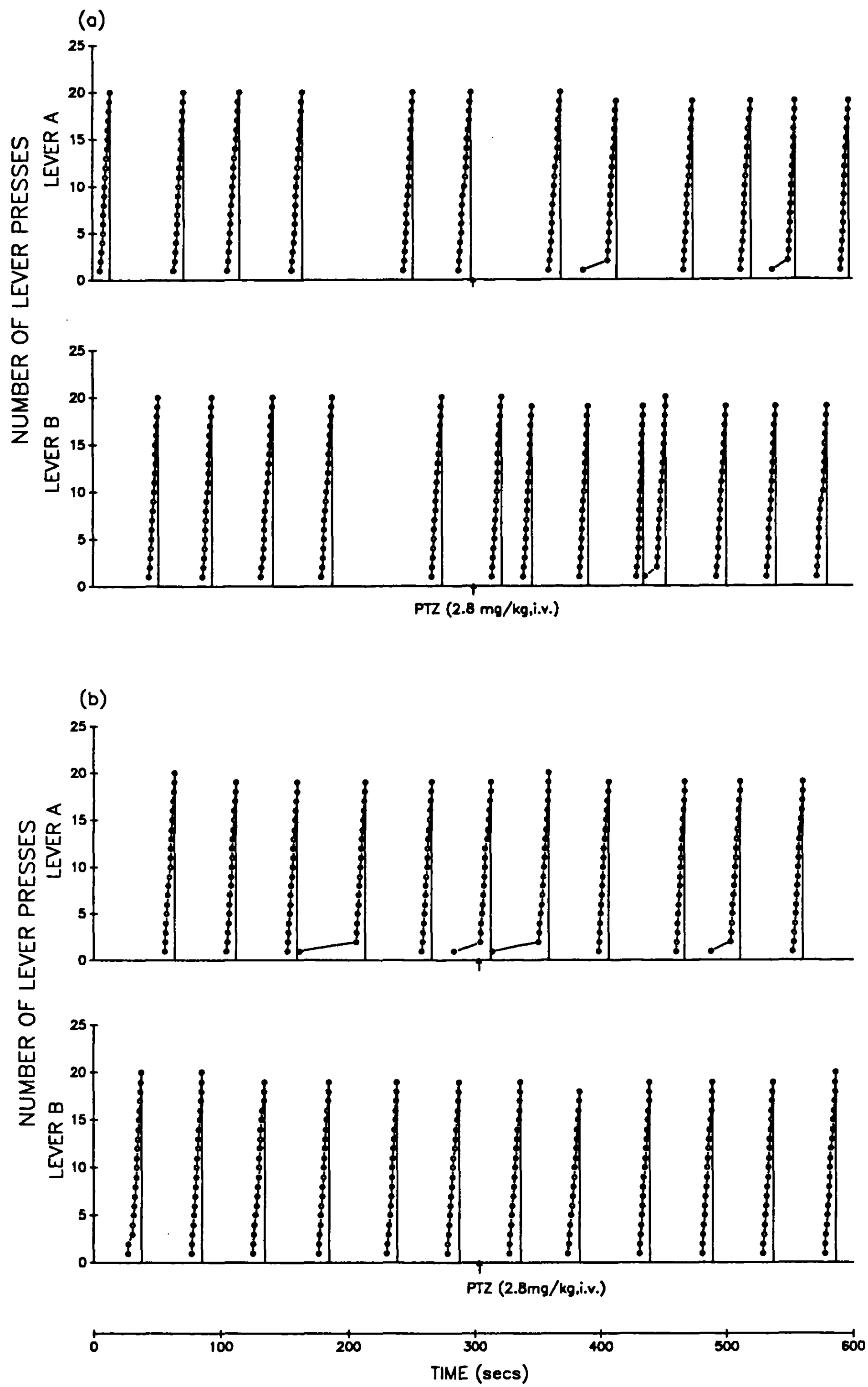


Fig 6.14 Cumulative records of the discriminatory response of pig C3 to an intravenous infusion of PTZ (5.256 mg/kg) following a saline or diazepam (0.5 mg/kg, p.o.) pretreatment: (a) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment, (b) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment and 30 min after a pretreatment with diazepam, (c) response to an infusion of PTZ 300 secs after a response to a saline pretreatment and 30 min after a treatment with the food vehicle alone.


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
PTZ (2.8 mg/kg,i.v.)
(b)
PTZ (2.8mg/kg,i.v.)
TIME (secs)

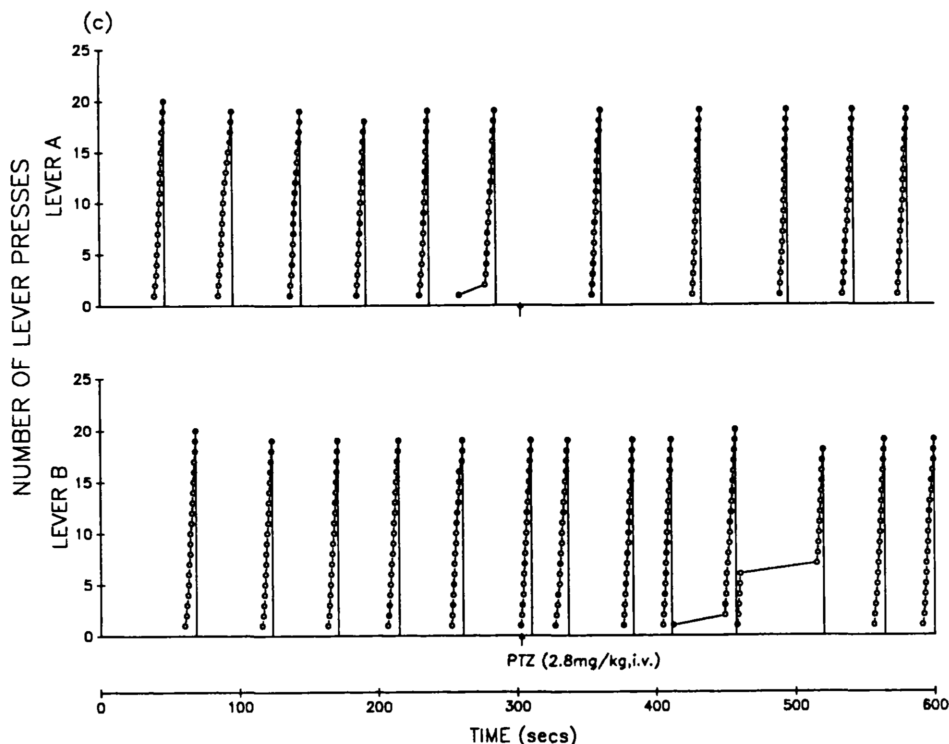


Fig 6.15 Cumulative records of the discriminatory response of pig C4 to an intravenous infusion of PTZ (2.8 mg/kg) following a saline or diazepam (0.5 mg/kg, p.o.) pretreatment: (a) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment, (b) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment and 30 min after a pretreatment with diazepam, (c) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment and 30 min after a treatment with the food vehicle alone.

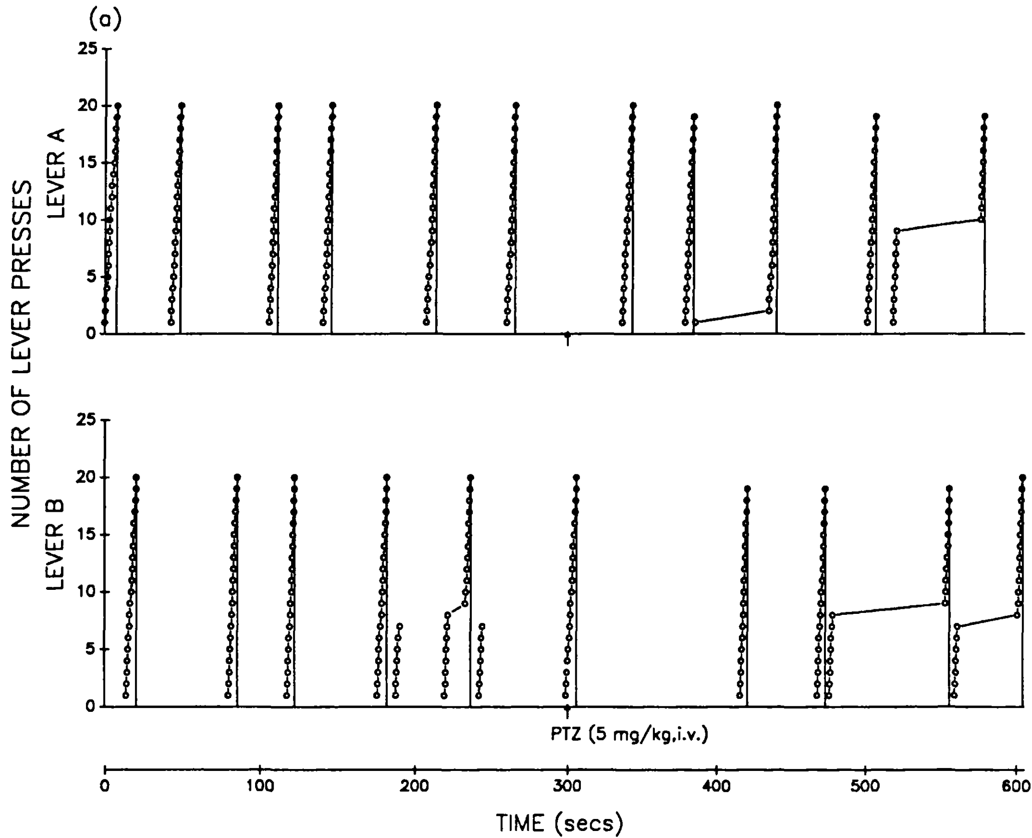
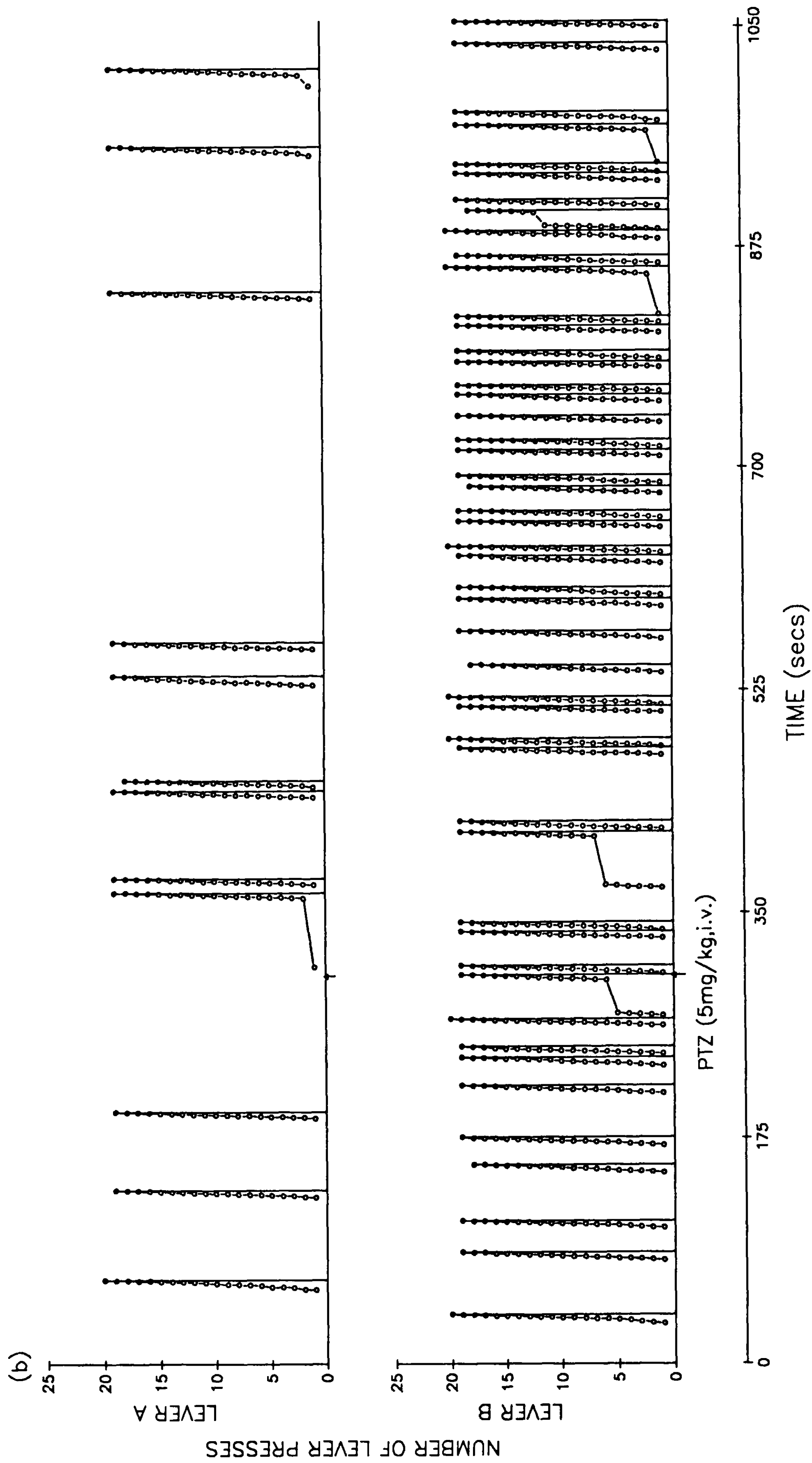


Fig 6.16 Cumulative records of the discriminatory response of pig C5 to an intravenous infusion of PTZ (5 mg/kg) following a saline or diazepam (0.5 mg/kg, p.o.) pretreatment: (a) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment, (b) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment and 30 min after a pretreatment with diazepam.



(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
25
20
15
10
5
0
PTZ (5mg/kg,i.v.)
TIME (secs)
0
175
350
525
700
875
1050

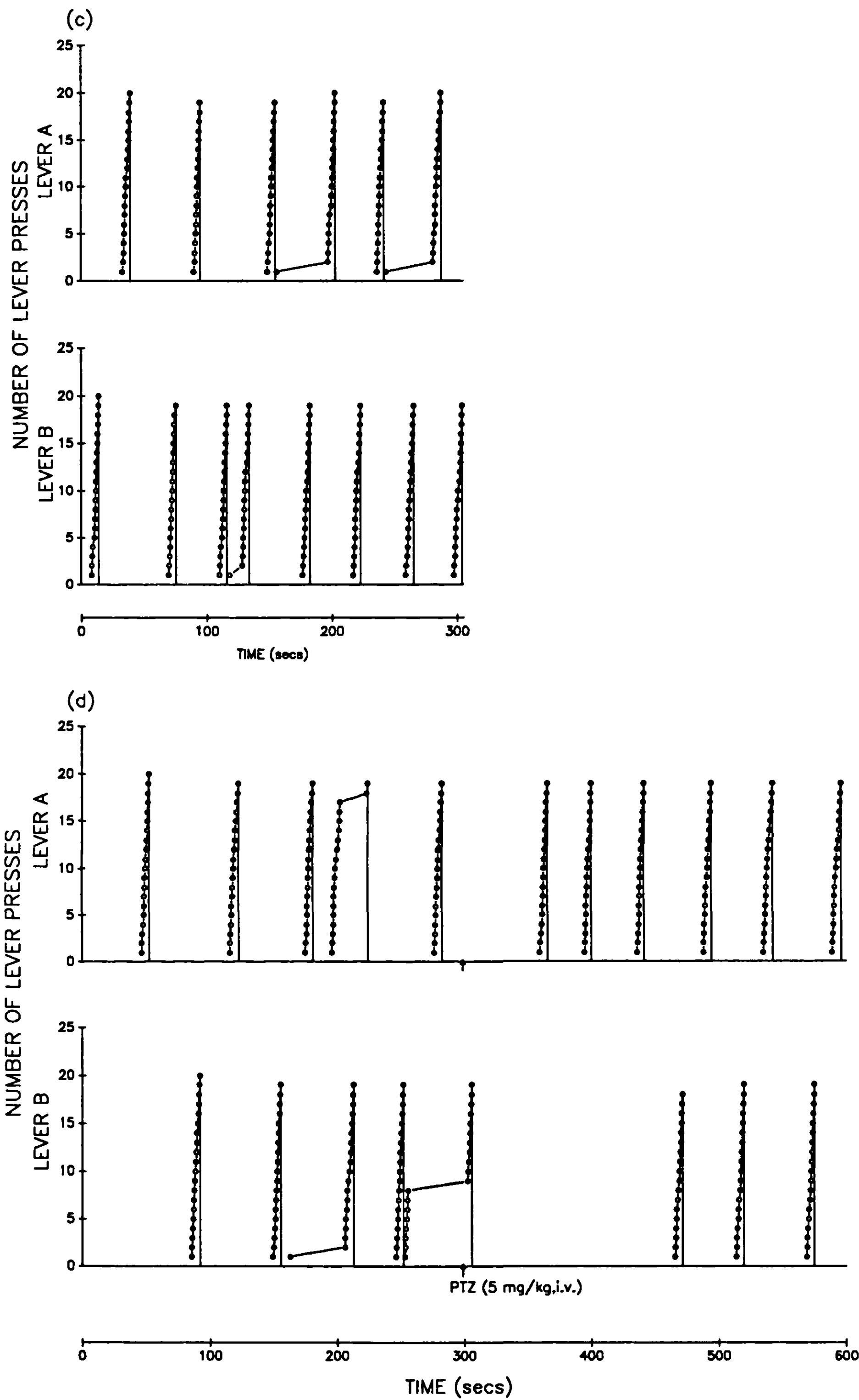


Fig 6.16 contd. (c) response to a saline pretreatment 73 min after diazepam administration, (d) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment and 30 min after a treatment with the food vehicle alone.

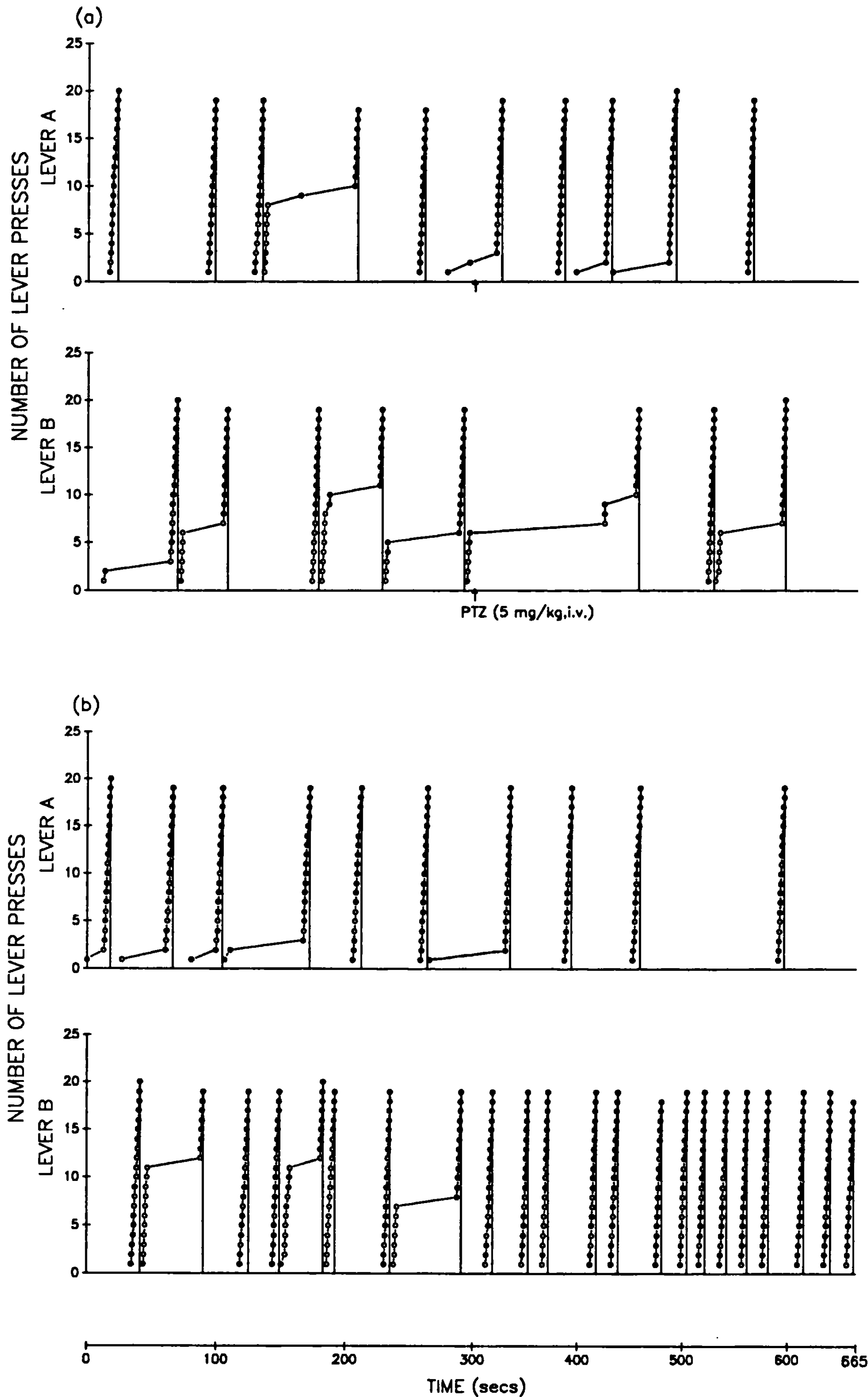


Fig 6.17 Cumulative records of the discriminatory response of pig C5 to an intravenous infusion of PTZ (5 mg/kg) following a saline or diazepam (0.5 mg/kg,p.o.) pretreatment: (a) response to an infusion of PTZ starting 300 secs after a response to a saline pretreatment, (b) effect of a diazepam pretreatment (30 min) on the response to an infusion of PTZ immediately before the test.

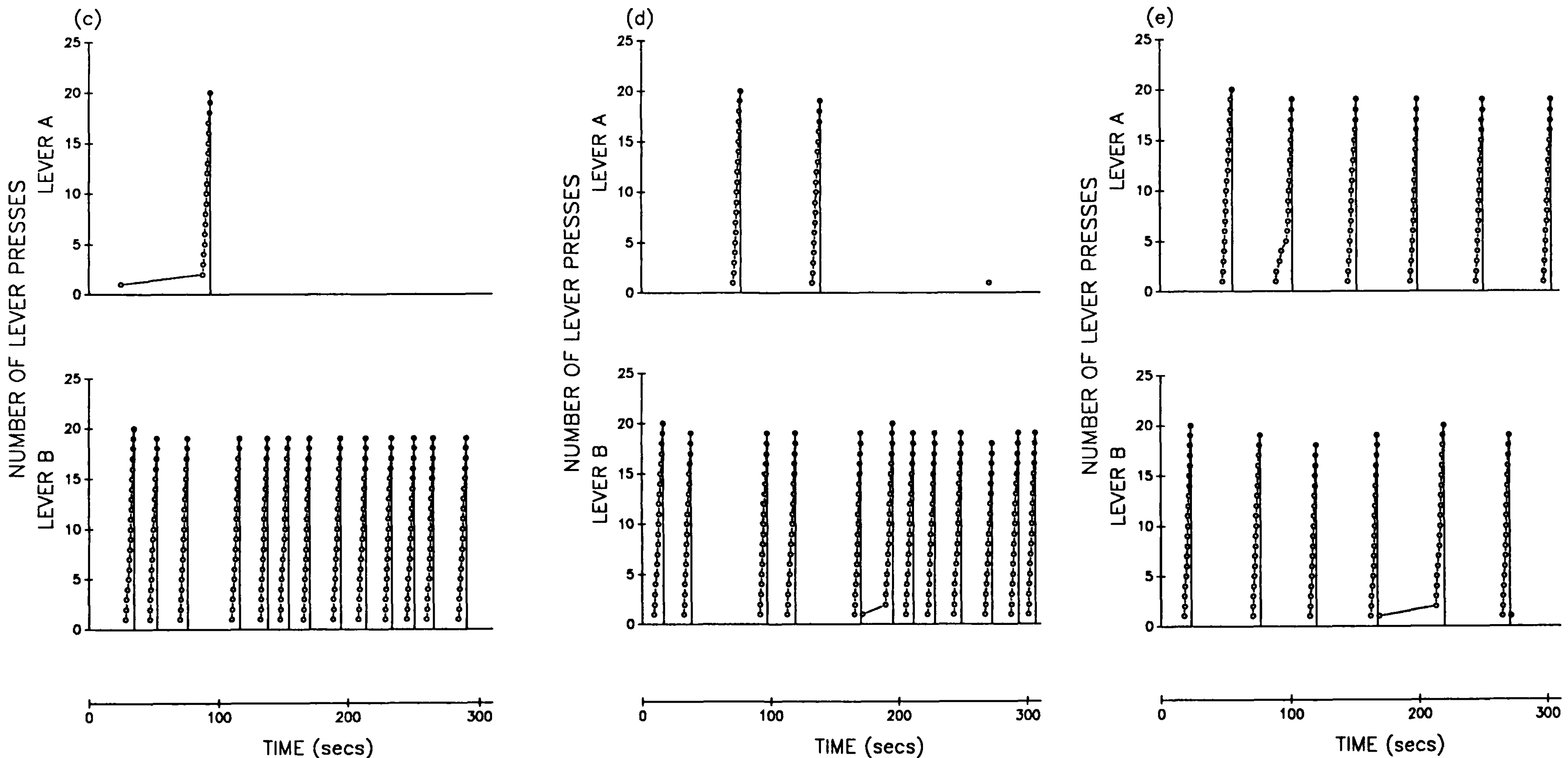


Fig 6.17 contd. response to a saline pretreatment at the following times after diazepam administration, (c) 71 min, (d) 106 min, (e) 141 min.

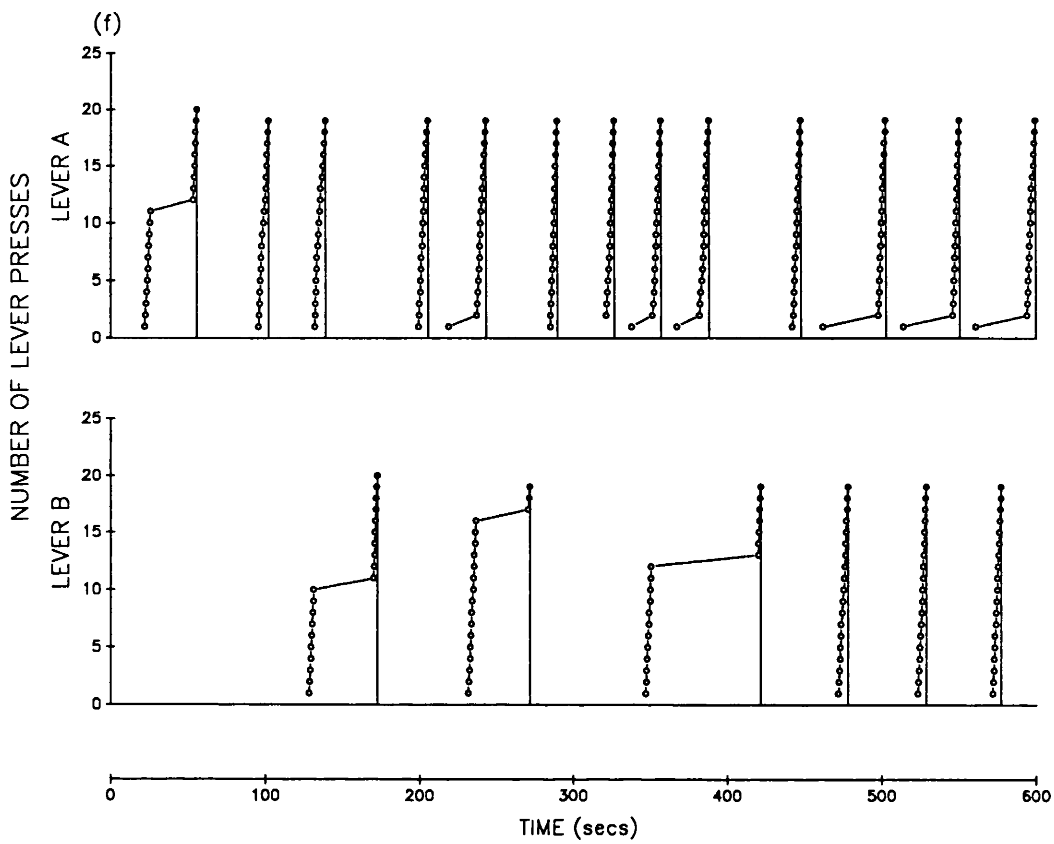


Fig 6.17 contd. (f) effect of a pretreatment with the food vehicle alone (30 min) on the response to an infusion of PTZ immediately before the test.

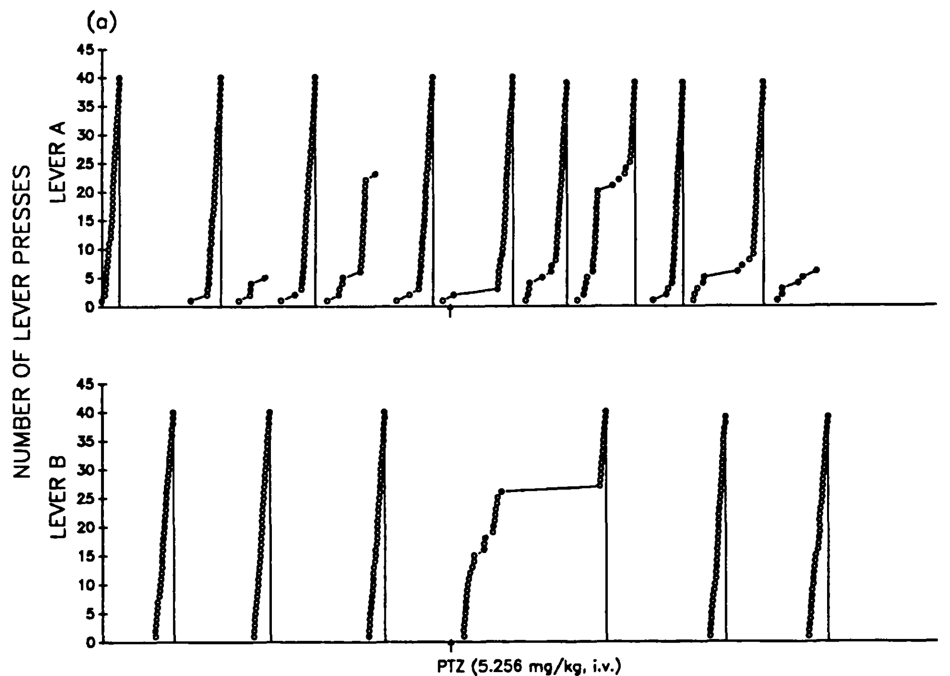
Effect of ethosuximide on the PTZ cue

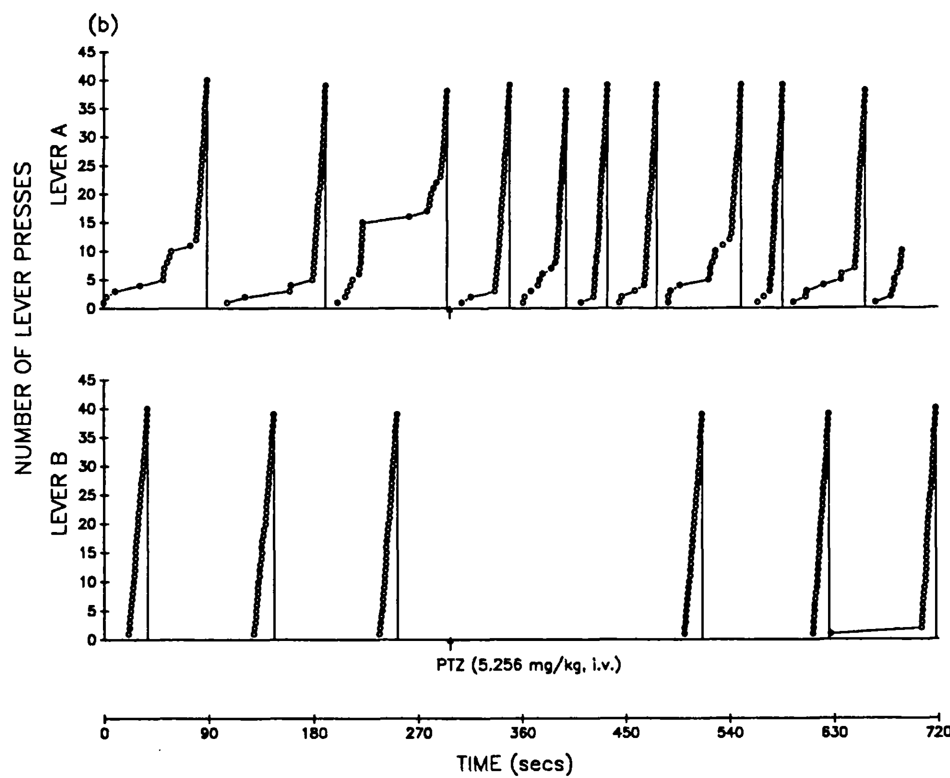
Infusion of the training dose of PTZ during a saline session, to pigs C3, C4 and C5 (pharmacologically conditioned by procedure 2B Chapter 2,Section 2.3.d), following pretreatment with ethosuximide (30 mg/kg,p.o.) resulted in a change in response from an alternate selection of both levers to a selection of the drug designated lever alone (Figs. 6.18b, 6.19b and 6.20b). The duration of this effect was 203.2 secs for pig C3, 81.3 secs for pig C4 and 114.1 secs for pig C5. A previous presentation of the training dose of PTZ to these pigs during a saline session produced a selection of the drug designated lever alone for a period of 130.2 secs for pig C3, 52.1 secs for pig C4 and 166.9 secs for pig C5 (Figs. 6.18a, 6.19a and 6.20a). Infusion of the training dose of PTZ to these animals during a saline session following treatment with the food vehicle alone, also resulted in a change in response from a selection of both levers alternately to a selection of the drug designated lever alone (Figs. 6.18c, 6.19c and 6.20c). The duration of this response was 126.4 secs for pig C3, 83.4 secs for pig C4 and 153.8 secs for pig C5.

From the above results, it is clear that ethosuximide did not antagonise the PTZ stimulus. This dose of ethosuximide was then tested for its ability to block PTZ induced convulsions in these pigs. PTZ doses of 7.88, 4.2 and 12.5 mg/kg, i.v. induced full seizures in pigs C3, C4 and C5 respectively. These seizures were characterised by a loss of righting reflex and the mean duration of this effect was 42.6 ± 3.72 secs. This was then followed by symptoms of body tremor, head twitch, salivation and extension of the forelimbs. Pretreatment of pigs C3 and C4 with ethosuximide (30 mg/kg, p.o.) completely prevented the above convulsant activity of PTZ. Following a pretreatment with ethosuximide (30 mg/kg, p.o.), PTZ (12.5 mg/kg, i.v.) induced proconvulsant behaviour in pig C5 characterised by extension of the forelimbs and jerking movements of the head. The duration of this activity was 36 secs.

In contrast to the report of Andrews et al. (1989), these results demonstrate that ethosuximide did not antagonise the PTZ cue at a dose which

was effective in preventing PTZ induced convulsions. This finding provides further confirmation that the PTZ cue in pigs corresponds to a state of anxiety.


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
45
40
35
30
25
20
15
10
5
0
PTZ (5.256 mg/kg, i.v.)


(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
45
40
35
30
25
20
15
10
5
0
PTZ (5.256 mg/kg, i.v.)
0
90
180
270
360
450
540
630
720
TIME (secs)

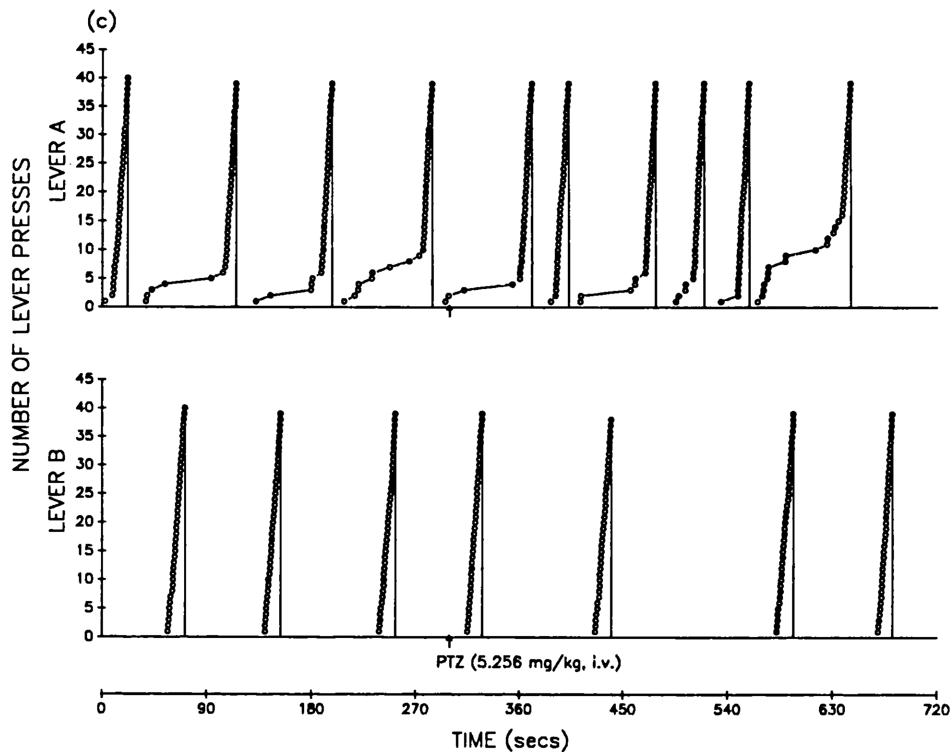
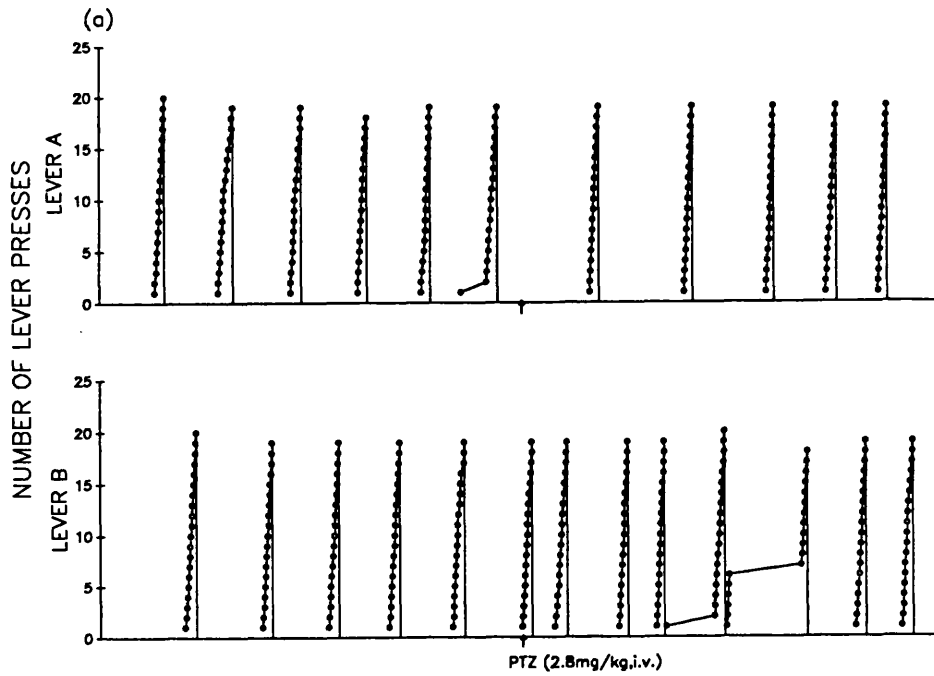
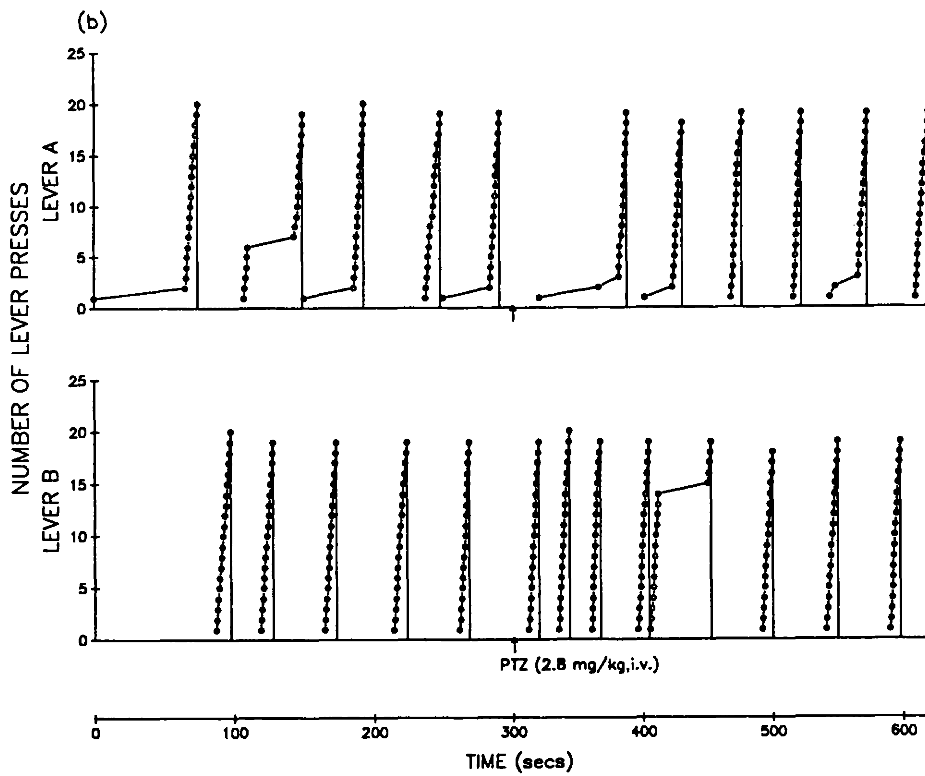


Fig 6.18 Cumulative records of the discriminatory response of pig C3 to an intravenous infusion of PTZ (5.256 mg/kg) following a pretreatment with saline, ethosuximide (30 mg/kg, p.o.) or the food vehicle control: (a) response to an infusion of PTZ 300 secs after a response to a saline pretreatment, (b) effect of a pretreatment with ethosuximide on the response to an infusion of PTZ 300 secs after a response to a saline injection, (c) effect of a treatment with the food vehicle alone on the response to an infusion of PTZ 300 secs after a response to a saline injection.


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
25
20
15
10
5
0
PTZ (2.8mg/kg,i.v.)


(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
25
20
15
10
5
0
PTZ (2.8 mg/kg,i.v.)
0
100
200
300
400
500
600
TIME (secs)

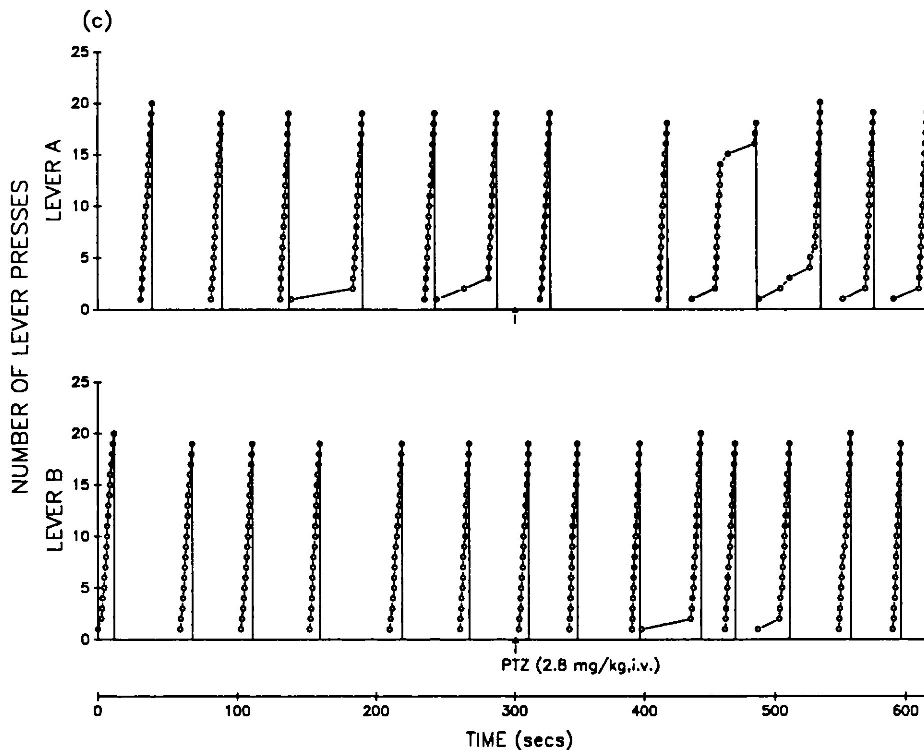
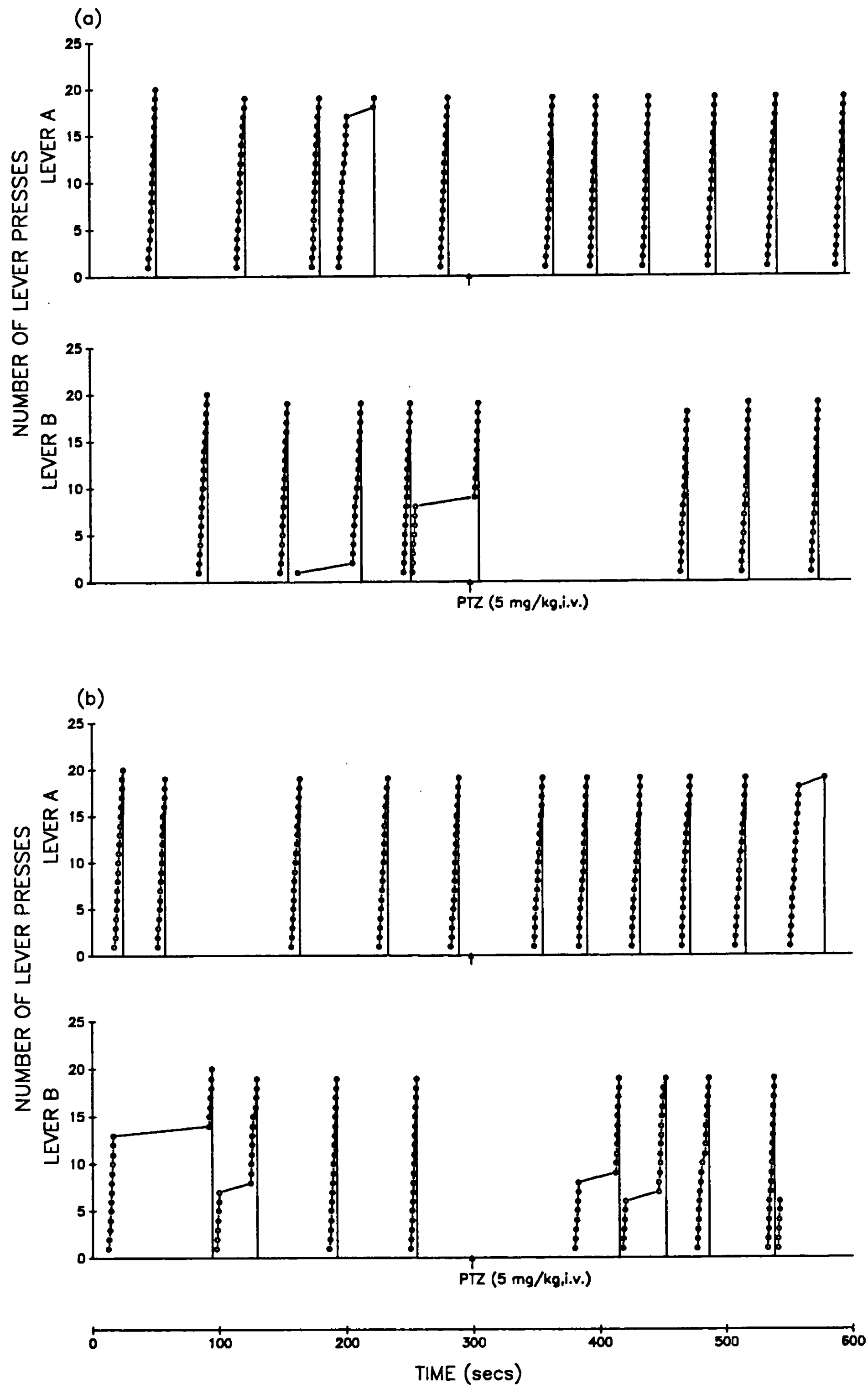


Fig 6.19 Cumulative records of the discriminatory response of pig C4 to an intravenous infusion of PTZ (2.8 mg/kg) following a pretreatment with saline, ethosuximide (30 mg/kg, p.o.) or the food vehicle alone: (a) response to an infusion of PTZ 300 secs after a response to a saline pretreatment, (b) effect of a pretreatment with ethosuximide on the response to an infusion of PTZ 300 secs after a response to a saline injection, (c) effect of a treatment with the food vehicle alone on the response to an infusion of PTZ 300 secs after a response to a saline injection.


(a)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
PTZ (5 mg/kg,i.v.)
(b)
NUMBER OF LEVER PRESSES
LEVER A
LEVER B
PTZ (5 mg/kg,i.v.)
TIME (secs)

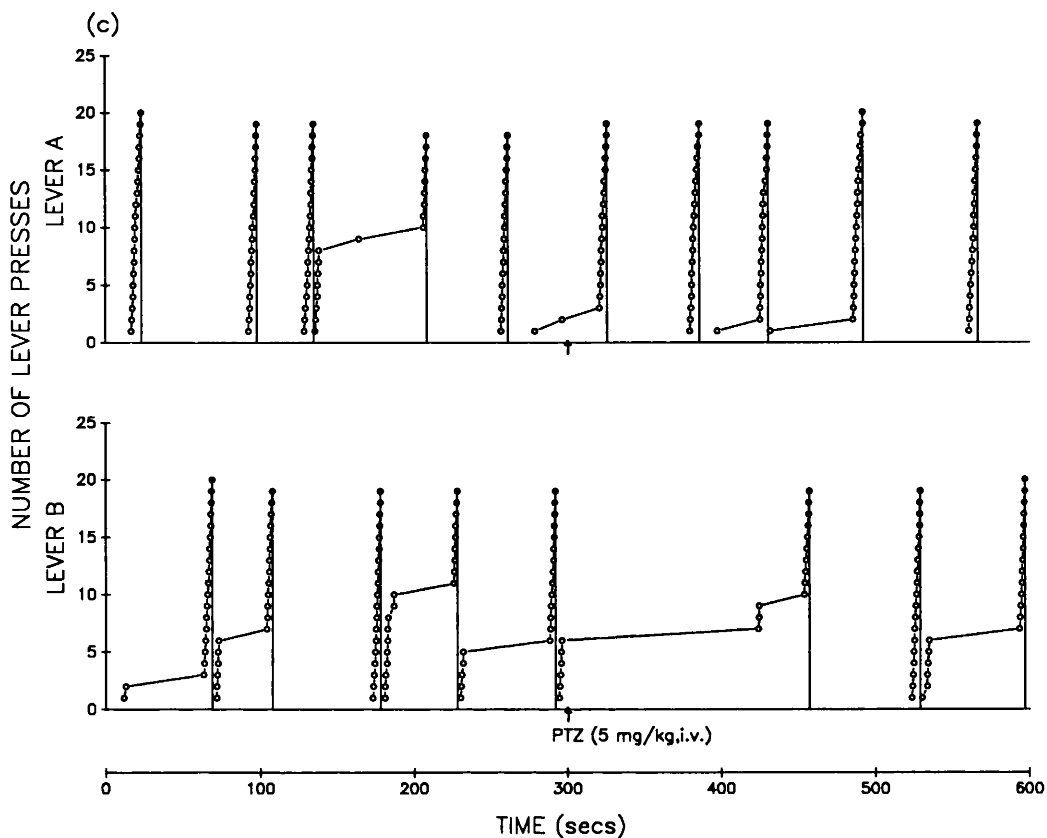


Fig 6.20 Cumulative records of the discriminatory response of pig C5 to an intravenous infusion of PTZ (5 mg/kg) following a pretreatment with saline, ethosuximide (30 mg/kg, p.o.) or the food vehicle alone: (a) response to an infusion of PTZ 300 secs after a response to a saline pretreatment, (b) effect of a pretreatment with ethosuximide on the response to an infusion of PTZ 300 secs after a response to a saline injection, (c) effect of a treatment with the food vehicle alone on the response to an infusion of PTZ 300 secs after a response to a saline injection.

6.3 Conclusions

The state induced by a conditioned anxiogenic stimulus was shown to generalise to the cue induced by PTZ in pigs. This generalisation was sensitive to antagonism by diazepam. These findings demonstrate that the PTZ discriminative stimulus in pigs corresponds to a state of anxiety. In addition, results from the present study have revealed that both a conditioned anxiogenic stimulus and an injection of PTZ summate and therefore appear to induce anxiety through a common mechanism: while a combination of a CES undergoing extinction and a subthreshold PTZ stimulus produced a generalisation to the PTZ cue, presentation of either of these stimuli alone did not result in an anxiogenic response.

In agreement with studies on the PTZ cue in rats (Stephens et al., 1984), diazepam was shown to specifically antagonise the PTZ stimulus in pigs. The ß-carboline FG7142 at a dose of 40 mg/kg was shown to produce a generalisation to the PTZ cue when tested in a pig that was pharmacologically conditioned by the conventional training procedure. An indication of an anxiogenic action of this dose of FG7142 was demonstrated only in one out of two pigs which were pharmacologically conditioned by the novel procedure described in the previous Chapter. These observations might be accounted for by the individual sensitivities of these animals to the anxiogenic action of FG7142 or by the complex pharmacology of this drug. In contrast to the findings of Andrews et al. (1989), ethosuximide did not antagonise the PTZ stimulus at a dose which prevented PTZ induced convulsions. This result provides further confirmation that the PTZ cue relates to a state of anxiety and not to a PTZ kindled preconvulsant aura.

From the above, it is clear that the PTZ discrimination paradigm in pigs is a valid model of anxiety. An experiment described in the present study suggests that this procedure is sensitive to the detection of a change in the psychological state of an animal on withdrawal from an acute treatment with a benzodiazepine tranquilliser. An enhanced sensitivity to the anxiogenic action of PTZ was observed in one pig, on the day following a treatment with

diazepam. In view of the important clinical implications of the dependency properties of benzodiazepine tranquillisers, the phenomenon of benzodiazepine withdrawal induced anxiety has been investigated in several animal analogues of anxiety (Emmett-Oglesby et al.,1983; Michaelis et al., 1988; Barrett & Smith, 1988; Baldwin & File, 1988).

In two such studies, rats were trained in a two lever operant procedure, to discriminate a 1,4-benzodiazepine from PTZ (Michaelis et al., 1988; Barrett & Smith, 1988). The animals were reinforced on a variable interval (VI) or variable ratio (VR) schedule and the vehicle condition was represented by a random response approaching a 50:50 ratio on the two levers. Such conditioning procedures allowed a ratio of PTZ to benzodiazepine response to be obtained in single sessions. It was, therefore, possible to demonstrate the time course over several sessions of the switch from a predominant benzodiazepine response to a predominant PTZ response following an acute treatment with a benzodiazepine. The conditioning procedure employed in the present study could also be applied to such an investigation. Animals could be trained to respond on one lever following a benzodiazepine treatment and on the other lever following PTZ treatment. The undrugged or vehicle condition would be represented by an alternation of lever selection response. The advantage of this procedure is that the undrugged state could be assessed very accurately and the time course of changes in the psychological state of the animal could be determined within a single session.

In addition to its potential usefulness in the study of benzodiazepine withdrawal induced anxiety, the present paradigm offers a reliable means, to investigate the neurochemistry and physiology of the anxiety state, to determine the anxiolytic or anxiogenic properties of clinical or veterinary therapeutic products and to evaluate putative anxiogenic interoceptive or exteroceptive stimuli e.g. hormonal fluctuations or various environmental stimuli (see Chapter 7).

Chapter 7

Application of the PTZ discrimination paradigm to assess aspects of pig welfare

7.1 Introduction

7.2 Results and discussion

7.2.a The discriminatory response of pigs to stimuli which mimic certain situations encountered in pig husbandry

7.3 Conclusions

7.1 Introduction

The welfare of an animal refers not only to its physical condition but also to its mental well-being (Brambell,1965). To date, the main methods employed to assess the variables which may induce psychological distress in farm animals have been; (1) Observations of the behaviour of animals under the given husbandry situation being considered. Any abnormalities detected have usually been taken as an indication of lowered welfare standards (Stephens, 1980). (2) Physiological changes that occur when animals are deliberately exposed to presumed stressful stimuli (Stephens & Toner, 1975; Stephens & Adams, 1982; Fry et al., 1981). Although, acknowledged to be an extremely valuable approach to the problem of welfare, the implications of any changes detected are not always clear. For example, during the act of suckling in the calf there is a profound reflex stimulation of the sympathetic nervous system (Stephens, 1982). (3) Behavioural conditioning techniques which allow self-selection of the environment by the animal (Dawkins, 1977; Stephens et al., 1985). Again, the precise interpretation of the observations and their implications in relation to the well-being of the animal is difficult (Duncan, 1978).

The PTZ discrimination paradigm described in the previous two chapters provides the pig with a means of expressing its own anxiety state and therefore, in contrast to the methods outlined above, this procedure permits a precise and reliable detection of the psychological state of the animal. The aim of the present study was to evaluate the anxiogenic properties of various pig husbandry situations by examining the discriminatory response of PTZ conditioned pigs to a range of putative aversive stimuli.

7.2 Results and discussion

7.2.a The discriminatory response of pigs to stimuli which mimic certain situations encountered in pig husbandry

The four pigs which were trained to discriminate the effects of PTZ from saline, by the novel conditioning procedure described and evaluated in the previous two chapters (and see Chapter 2, Section 2.3.d, Procedure 2B for details of training), were presented with a range of environmental stimuli and the subsequent discriminatory response of the animals was assessed. The environmental stimuli were selected so as to incorporate the following variables of pig husbandry systems: interaction with other pigs, transport, abattoir conditions, changes in temperature, the presence of putative threatening stimuli, the availability of food and the introduction of novel stimuli. PTZ/saline training sessions were interposed between the above tests to ensure that the PTZ discriminatory response was maintained.

Interaction with other pigs

When strange male pigs are housed together, fighting usually ensues until a social hierarchial structure is formed whereby the heaviest pig is usually the most dominant and the smallest the least dominant (Signoret et al., 1975; Lean, 1988).

In the present study, pigs C1, C3, C4 and C5 were mixed for a period of less than 10 mins with two strange prepubertal male pigs, to examine the possibility of a psychological disturbance in these animals following the brief encounter. In order to carry out this experiment the pigs were subjected to a 5 min test in the Skinner box following a saline pretreatment. They were then returned to their home pens to encounter the two strange pigs and following this encounter they were retested. The results obtained from this experiment for pigs C4 and C1 are shown in Fig. 7.1. Prior to the encounter, the animals selected both levers alternately. This was also the case for pigs C3 and C5 (results not shown). In all four cases, the encounter of the test pig with the two strange pigs resulted in agonistic behaviour (e.g. Fig. 7.2). Following the

encounter, pig C4 made two consecutive selections of the drug designated lever (lever B; Fig. 7.1.a). These selections were made at the start of the test and the duration of this response period was 59.6 secs. The alternate lever was then selected and this was followed by a further two consecutive selections of the drug designated lever for a response period of 45.3 secs. The animal then selected both levers alternately for the remainder of the 5 min test period.

After the encounter with the strange pigs, the response of pig C1 consisted initially of an alternate selection of both levers (Fig. 7.1.b). However, after a period of 234.5 seconds the animal began a selection of the drug designated lever (lever A) which was followed by three consecutive selections of this lever. The duration of this response period was 80.6 secs. Following this period of drug lever selection, a stable alternation of lever selection response was not observed; there were two further occurrences of consecutive selections of the drug designated lever and the test was terminated after 500 secs because the animal stopped responding and turned away from the response panel to face the exit of the box. The encounter with the strange pigs did not affect the alternation of lever selection response of pigs C3 and C5 in subsequent sessions.

The above results show that when strange male pigs are mixed together, a state of anxiety is induced in some of these animals. The failure to detect an anxiogenic response in pigs C3 and C5 may reflect a difference in the order of dominance of these pigs. It is possible that anxiogenic effects induced by the encounter were overcome by placing the animals in familiar surroundings (i.e. Skinner box in the conditioning room) with no associations of threat or fear. It is clear from Fig. 7.1a that in this familiar situation, pig C4 showed a rapid recovery from an anxiogenic state.

The observed anxiogenic response to an encounter with strange pigs could have resulted from the interaction of fighting and/or from the threat which the presence of the strange animals may have imposed. The latter possibility was investigated by examining the response of pigs C1, C4 and C5 to the presence of a preputial gland secretion from a strange boar. It is known

that olfaction plays a significant role in social interaction among pigs and the preputial gland is responsible for the characteristic odour of the boar (Signoret et al., 1975).

The preputial gland secretion was evenly rubbed on the front panel of the Skinner box but not on either of the two levers or the food bowl. In addition a beaker of this fluid was placed under the Skinner box equidistant from both levers. Fig. 7.3 shows the effect of the preputial gland secretion on the response of pigs C1 and C5. This stimulus produced an anxiogenic response in pig C1 whereby the animal initially responded on the drug designated lever only, for a period of 86.5 secs. The alternate lever was then selected but this selection was followed by a further pair of consecutive selections of the drug designated lever. A stable alternation of lever selection response then ensued until the end of the 10 min test period. Presentation of the preputial gland secretion to pig C5 (Fig. 7.3b) and pig C4 (result not shown) resulted in a selection of both levers alternately. These results indicate that pig C1 was affected not only by the physical interaction with a strange pig but also by the smell of such an animal. An experiment was then conducted to investigate the specificity of this effect to the sex of the strange pig.

The discriminatory response of pigs C1, C3, C4 and C5 to the presence of urine obtained from a gilt in oestrus, was examined. As for the presentation of the preputial gland secretion, the urine was evenly rubbed on the front panel of the box and a piece of straw saturated in urine was placed under the front of the Skinner box, equidistant from both levers. Presentation of this stimulus to pig C1 resulted in selections of the drug designated lever only for a period of 121.9 secs at the start of the test. Following this period, a stable alternation of lever selection response was observed (Fig. 7.4a). This stimulus also induced an anxiogenic response in pig C3 (Fig. 7.4b). Although, the initial response of this animal consisted of an alternate selection of both levers, after a period of 266.4 secs the animal began a selection of the drug designated lever which was followed by an exclusive response on this lever for a duration of 118.8 secs. The presentation of the gilt urine stimulus to pigs C4 and C5 produced an alternation of lever selection response (result not shown).

The specificity of the observed responses to the above stimuli was examined by presenting the animals with a non-specific olfactory stimulus. A beaker of thymol, freshly melted in hot water was placed under the front of the Skinner box equidistant from both levers and the discriminatory response of pigs C1, C3, C4 and C5 to the presence of this stimulus was examined. In contrast to the preputial gland secretion and the gilt urine, thymol did not induce an anxiogenic response in any of the animals (result not shown).

The above results indicate that the mixing of strange pigs, either male pigs with male pigs or male pigs with female pigs, may induce a state of anxiety irrespective of whether any physical contact such as fighting takes place. It is unlikely that the anxiogenic response of pigs C1 and C3 to the presentation of the gilt urine, represents a frustrated sexual reaction since the detection of oestrus females by male pigs appears to occur primarily in response to visual stimuli rather than smells (Signoret et al., 1975) and at the time of testing, these male pigs were several weeks younger than the typical age at which pigs attain puberty. From Figs. 7.3 and 7.4 it is clear that a continuous exposure to the aversive olfactory cues associated with a strange boar or gilt, resulted in a rapid habituation of the anxiogenic response to these stimuli. It is possible, therefore, that in husbandry situations an exposure to such stimuli prior to mixing with strange pigs might alleviate some of the resultant aberrant behaviour.

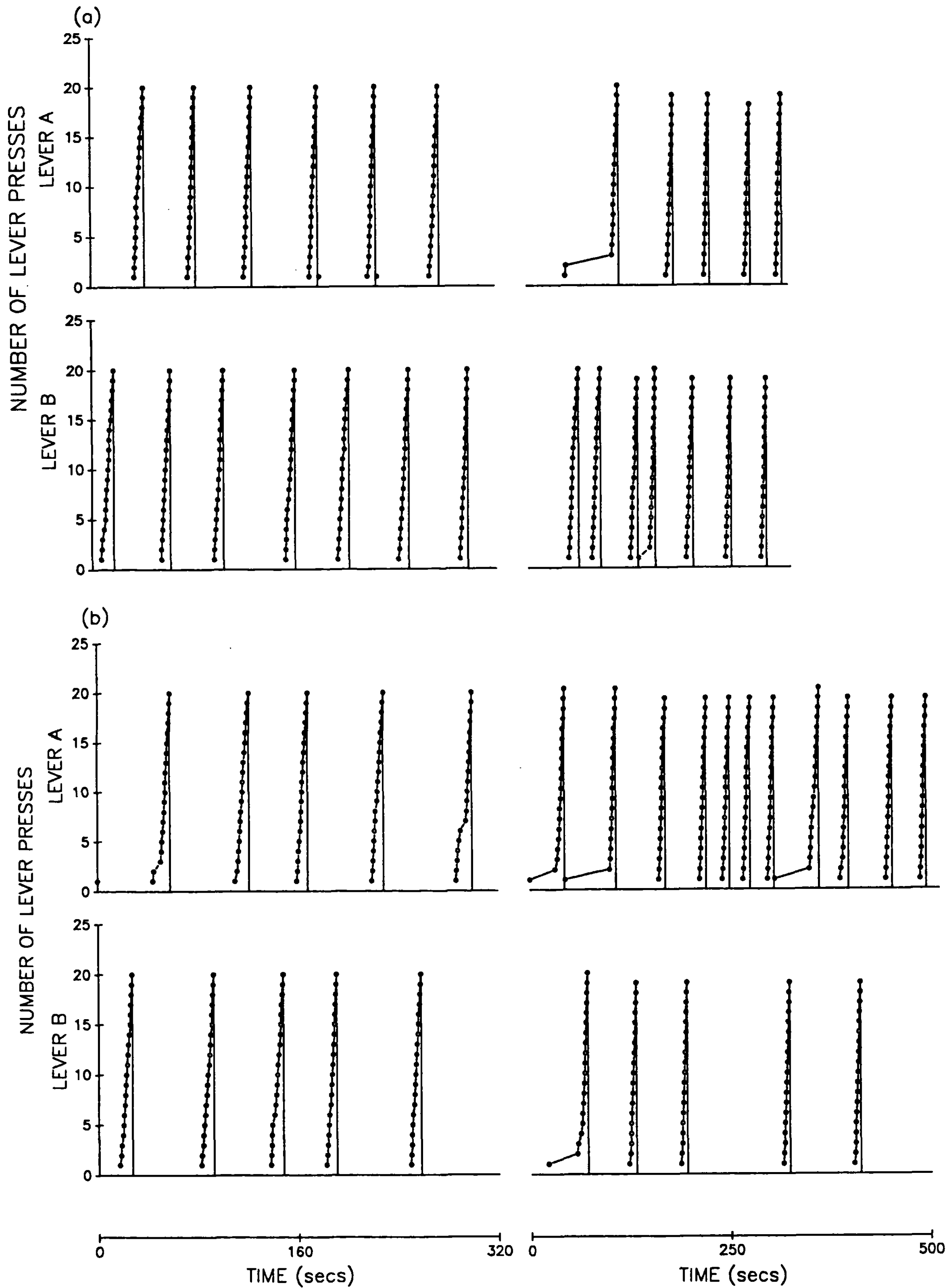


Fig. 7.1 Cumulative records of the discriminatory response of pigs before and after an encounter in the home pen with two strange pigs: response of pig C4 (a; PTZ lever=B) and pig C1 (b; PTZ lever=A), after an injection of saline and the subsequent response following the encounter with the strange pigs.

Fig. 7.2 Interaction of test pig with two strange male prepubertal pigs.

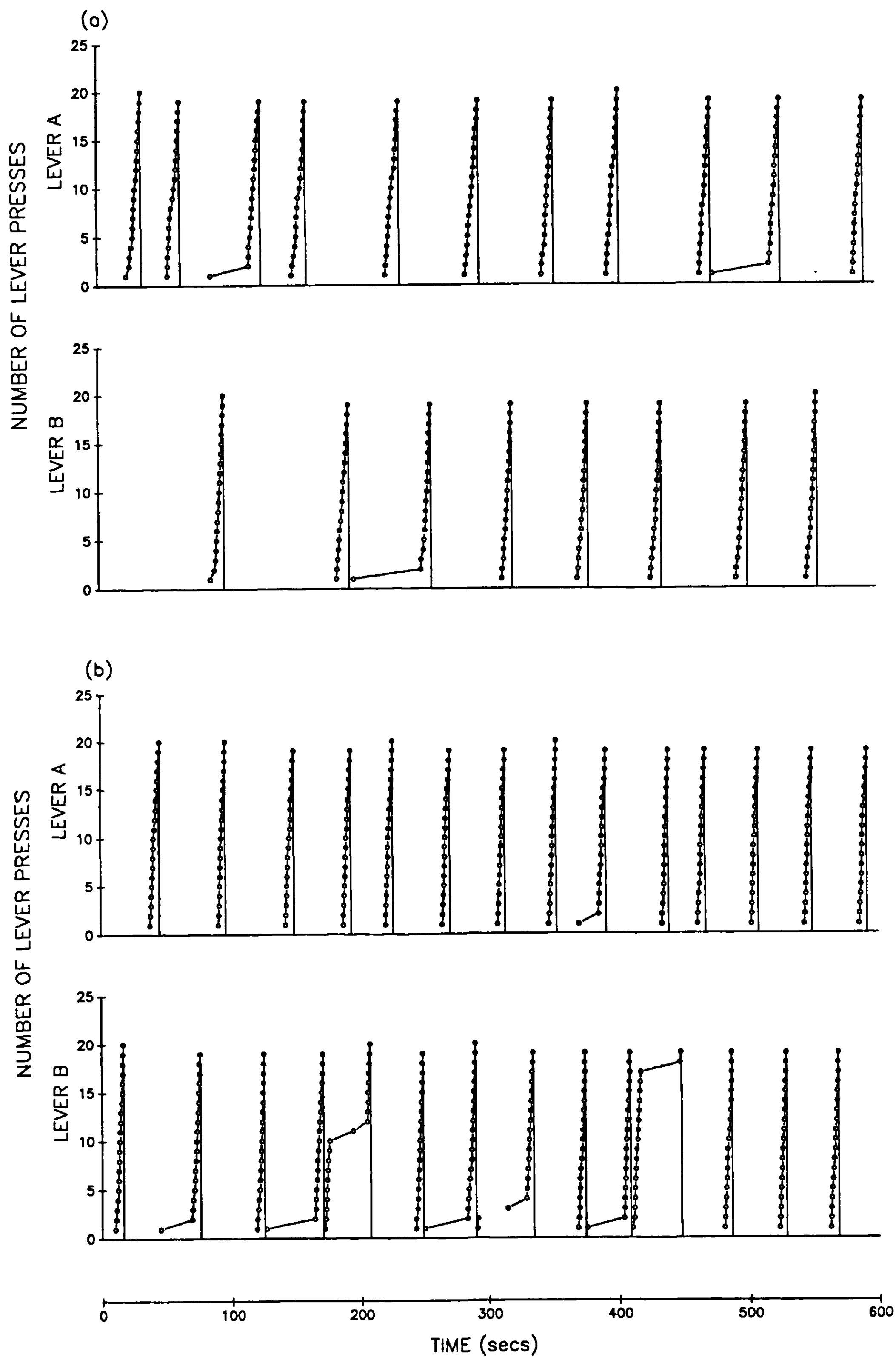


Fig. 7.3 Cumulative records of the discriminatory response of pigs to the smell of the secretion from the preputial gland of a strange boar: (a) response of pig C1 (PTZ lever=A), (b) response of pig C5 (PTZ lever=A).

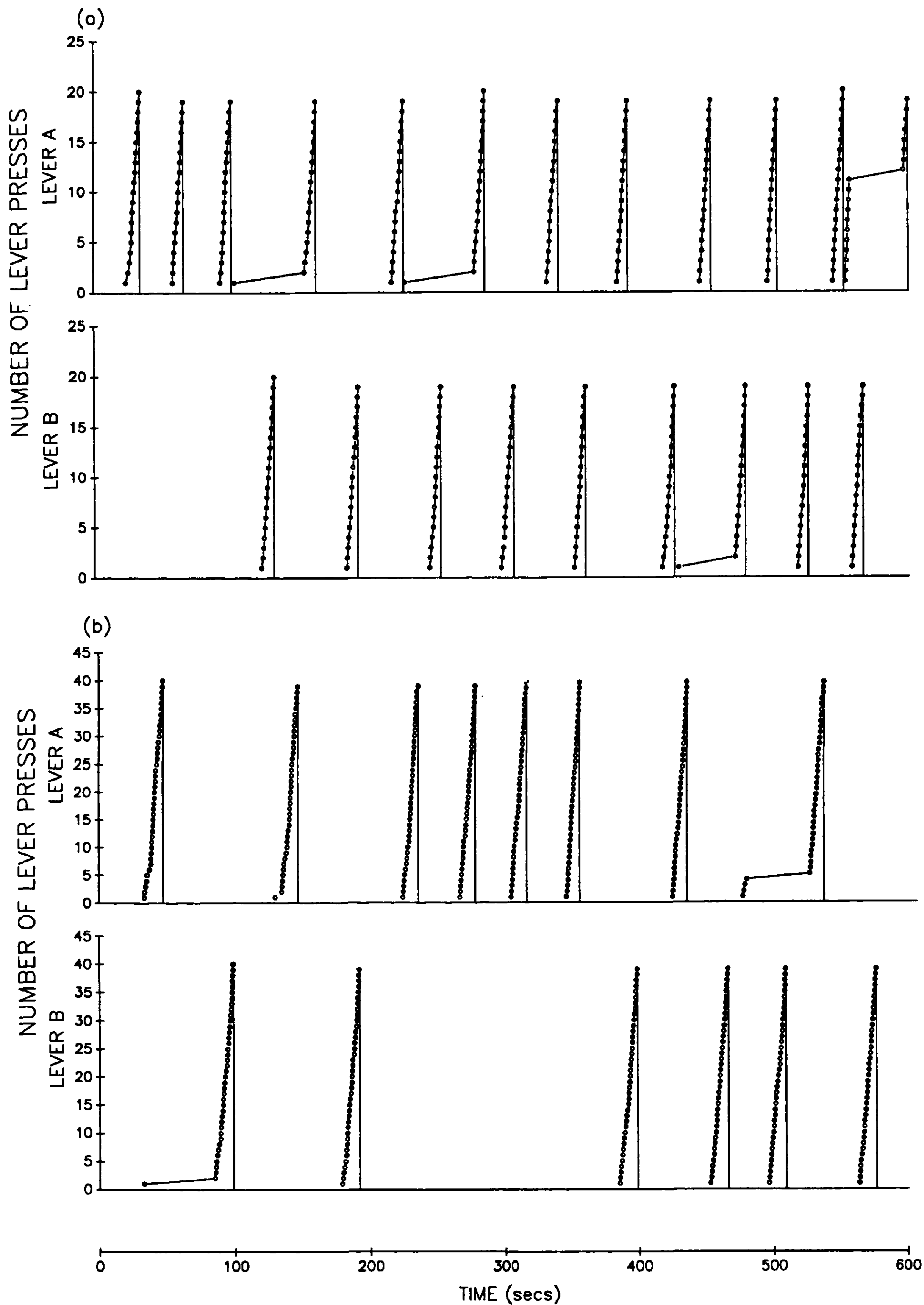


Fig. 7.4 Cumulative records of the discriminatory response of pigs to the smell of urine obtained from a gilt in oestrus: (a) response of pig C1 (PTZ lever=A), (b) response of pig C3 (PTZ lever=A).

Transport

It is well known that pigs die from stress during the process of transportation (e.g. see Allen, 1979). In an attempt to define the stressful factors of this process, Stephens et al. (1985) simulated the vibration and noise components of transport and results from this study suggested that vibration was aversive to these animals but noise was not. In the present study, an attempt was made to investigate the effect of a short journey on the anxiety state of the pig.

The pigs were transported individually in a trailer hitched to a LandRover, which was driven at a speed of 10-30 mph for a period of 20 minutes. Immediately after this journey, the pig was removed from the trailer and tested in the Skinner box. The result obtained from this experiment for pig C4 is shown in Fig. 7.5a. A stable alternation of lever selection response did not occur until after a period of 237.4 seconds. During this period the animal made 12 selections of the drug designated lever and only one selection of the alternate lever. In contrast to this result, an anxiogenic effect of transportation was not evident in pig C5 (Fig. 7.5b) or pig C3 (result not shown). Again, the failure to detect an anxiogenic response in these pigs maybe because the tests were conducted in a familiar non-aversive environment. A more reliable assessment of the anxiety inducing properties of transportation might be obtained by examining the discriminatory response of these animals during the transport process.

Abattoir conditions

The possibility that the smell of freshly killed pig carcasses at an abattoir might be anxiogenic to pigs awaiting slaughter was investigated.

A carcass of a freshly killed pig was placed in the conditioning room at the front of the Skinner box. The carcass was in a position such that it could not be seen from within the Skinner box. The four pigs C1, C3, C4 and C5 were then subjected to a 10 min test with the carcass left in place. The results of this experiment for pigs C3 and C1 are shown in Fig. 7.6. The drug designated lever was selected by pig C3 for a period of 156.7 secs at the start of the test

and this was followed by an alternation of lever selection response for the remainder of the 10 min test period. An anxiogenic response to the smell of the carcass was also evident in pig C1 whereby three consecutive selections were initially made on the drug designated lever. The duration of this response period was 215.8 secs. For the remainder of the test period, the animal selected both levers alternately with one occurrence of two consecutive selections of the drug designated lever. The presence of the pig carcass had no effect on the alternation of lever selection response of pigs C4 and C5 (results not shown).

To ensure that the order of testing did not influence the detection of an anxiogenic state, the experiment was repeated with a fresh pig carcass and the animals were tested in a reverse order. On this occasion, the presence of the pig carcass did not induce an anxiogenic response in any of the animals.

The above results demonstrate a rapid habituation to the anxiogenic properties of an aversive stimulus induced by a short term exposure to the stimulus.

Changes in temperature

The effect of a dramatic reduction in ambient temperature on the psychological state of the pig was examined. The temperature of the conditioning room was decreased from its normal temperature of 20-22°C to 5°C. Pigs were removed from their home pens which were maintained at a temperature of 18-20°C and were then tested in the conditioning room at the reduced temperature of 5°C.

An anxiogenic response to the cold stimulus was observed in pigs C1 and C5. The drug designated lever was selected by pig C1 for the initial 107.5 sec period of the test. This was followed by an alternation of lever selection response for the remainder of the test period (Fig. 7.7a). The drug designated lever was selected by pig C5 for the initial 58.9 secs of the test and then the animal selected both levers alternately to the end of the 10 min test period (Fig 7.7b). The change in temperature did not induce an anxiogenic response in pigs C3 and C4 (results not shown).

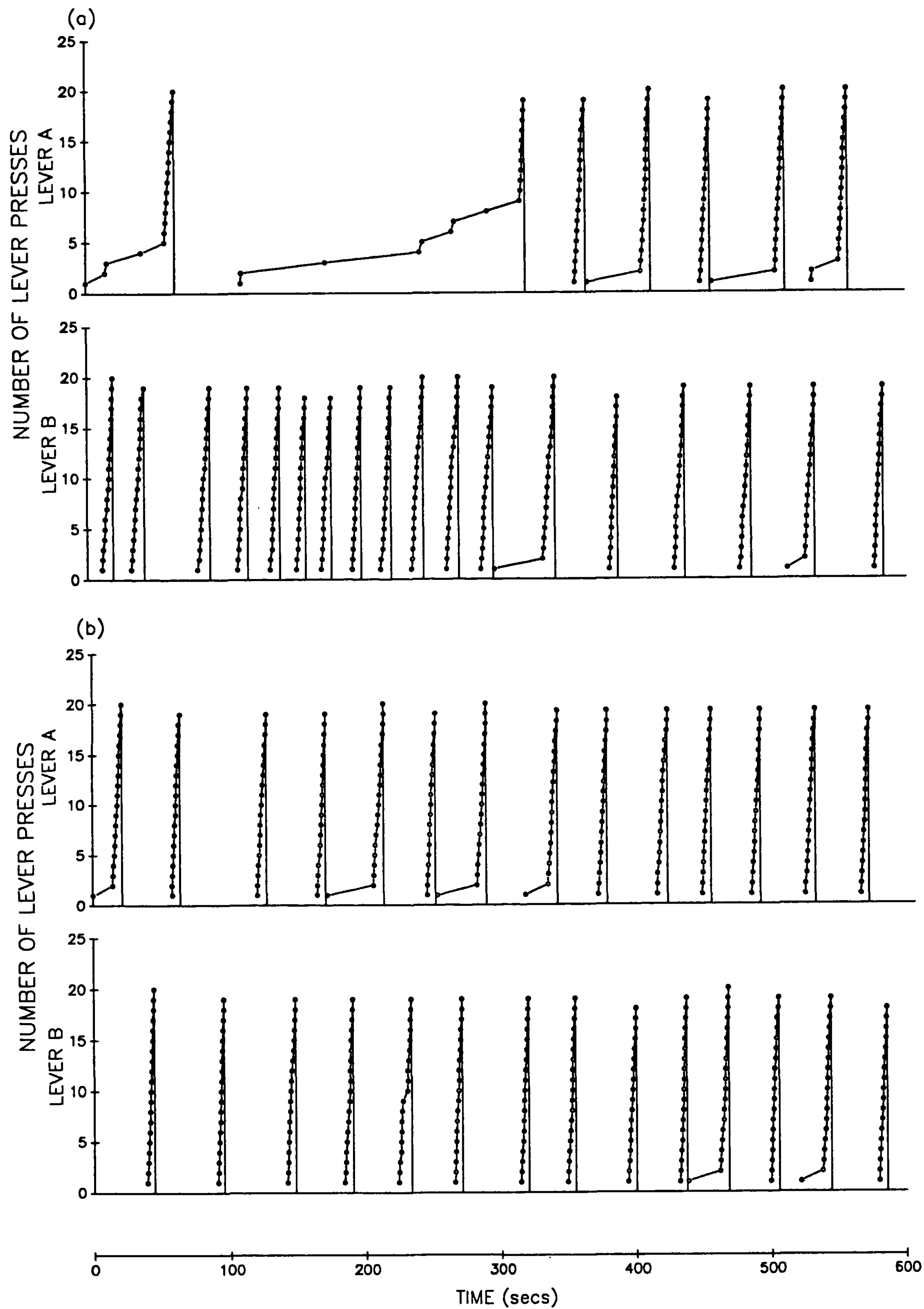


Fig. 7.5 Cumulative records of the effect of individual transportation for 20 min in a trailer on the subsequent discriminatory response of pigs: (a) response of pig C4 (PTZ lever=B), (b) response of pig C5 (PTZ lever=A).

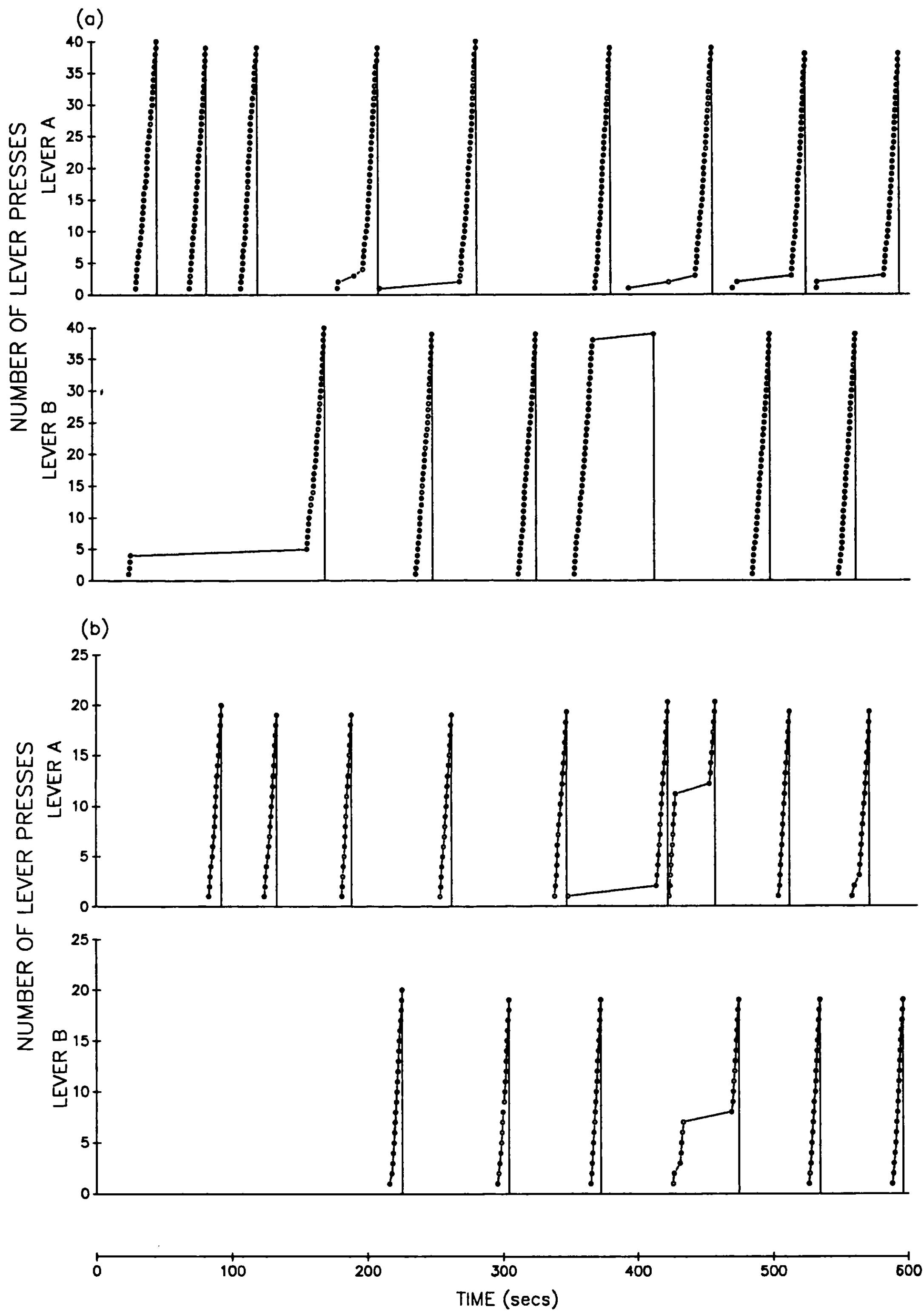


Fig. 7.6 Cumulative records of the discriminatory response of pigs to the presence of a freshly killed pig carcass: (a) response of pig C3 (PTZ lever=A), (b) response of pig C1 (PTZ lever=A).

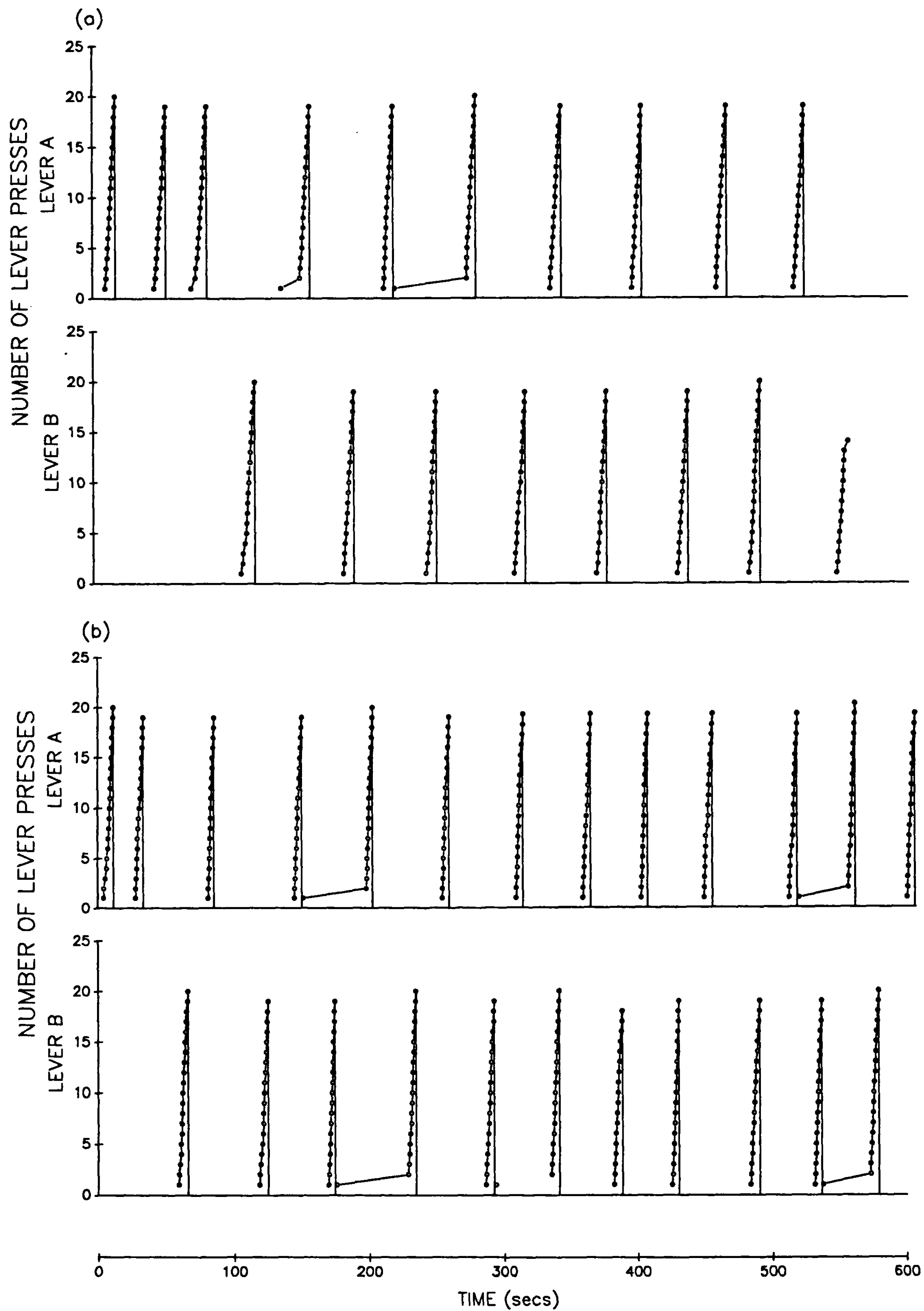


Fig. 7.7 Cumulative records of the effect of a decrease in ambient temperature on the discriminatory response of pigs: (a) response of pig C1 at 5°C (PTZ lever=A), (b) response of pig C5 at 5°C (PTZ lever=A).

The presence of putative threatening stimuli

When pigs are handled or when attempts are made to restrain the animals, they respond with high-pitched squealing. It is possible that such vocalisations function as a signal to warn other pigs of the presence of potentially dangerous stimuli. In view of this possibility, the effect of such a vocalisation on the discriminatory response of three pigs was examined.

A pig was restrained by the hind legs and the subsequent vocalisations of this animal were recorded. Such handling procedures are commonly encountered in pig husbandry (e.g. for the administration of injections). Pigs C1, C4 and C5 were pretreated with saline and following 5 mins of an alternation of lever selection response, the tape recording of the squealing pig was presented for a duration of 100 secs.

The results of this experiment for pigs C1 and C4 are shown in Fig. 7.8. These animals selected both levers alternately before the tape recorder was turned on. During the tape playback, a total suppression of response was observed for pig C1. This suppression of response was maintained for a period of approximately 380 secs after the tape recorder was turned off. At this time, the animal selected the drug designated lever and following this selection the test was terminated as the pig turned around in the box to face the exit. At the onset of the tape playback, pig C4 switched from pressing the A lever to pressing the drug designated lever. Following this lever selection, an alternation of lever selection response was observed for the remainder of the test period. During the tape playback a suppression of response was observed for pig C5 but this animal showed an alternation of lever selection response before and after the tape playback (result not shown).

While the above results do not demonstrate an anxiogenic response to the vocalisations of a restrained pig, they do indicate a psychological disturbance induced by such vocalisations.

The anxiety-inducing effect of the bark of a dog was then examined since this stimulus is known to induce fear in other farm animals and it may incite

some innate fear of a predator. The effect of a tape recording of a barking dog on the discriminatory response of pigs C1 and C4 are shown in Fig.7.9. During the 89 secs of the tape playback, a suppression of response was observed in pig C1. The animal did not resume response until 143.8 secs after the tape recorder was turned off. This response consisted of three consecutive selections of the drug designated lever. The duration of this response was 124.6 secs. The alternate lever was then selected but this was followed by a further period of response on the drug lever only, for a duration of 105.9 secs. A stable alternation of lever selection response did not occur until approximately 460 secs after the tape recorder was turned off. During the tape playback, a suppression of response was observed for pig C4 (Fig 7.9b) and pig C5 (result not shown) but these animals showed an alternation of lever selection response before and after the tape playback.

In an attempt to provide a control auditory stimulus for the squealing pig and barking dog stimuli, a recording was made of the sounds present in the barn of the test pigs when these animals were undisturbed. Presentation of this recording to pig C4 resulted in an alternation of lever selection response (result not shown). However, this stimulus caused a slight disruption of the stable alternation of lever selection response of pig C5 whereby the animal made two consecutive selections of the B lever (non-drug lever) after the onset of the tape playback (result not shown). Nevertheless, in contrast to the results obtained from the presentation of the recordings of the squealing pig and barking dog, the present recording did not induce an anxiogenic response or a suppression of response in either of the two pigs tested. This finding, therefore, suggests that the behaviour observed in response to the auditory stimuli of squealing pig and barking dog reflects a psychological disturbance specific to these stimuli.

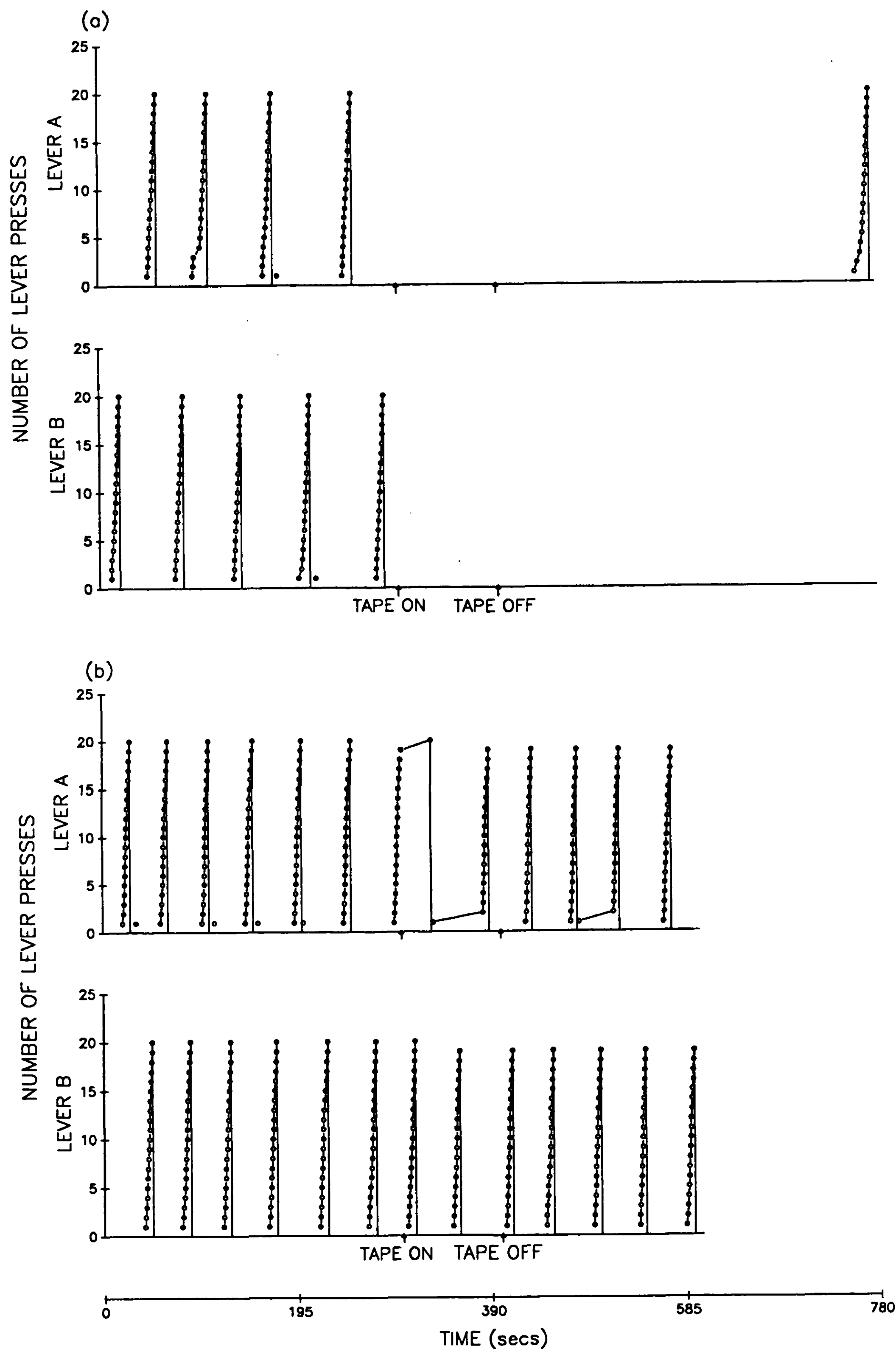


Fig. 7.8 Cumulative records of the discriminatory response of pigs presented with a tape recording of a squealing pig of duration 100 secs starting 300 secs after a response to a saline pretreatment: (a) response of pig C1 (PTZ lever=A), (b) response of pig C4 (PTZ lever=B).

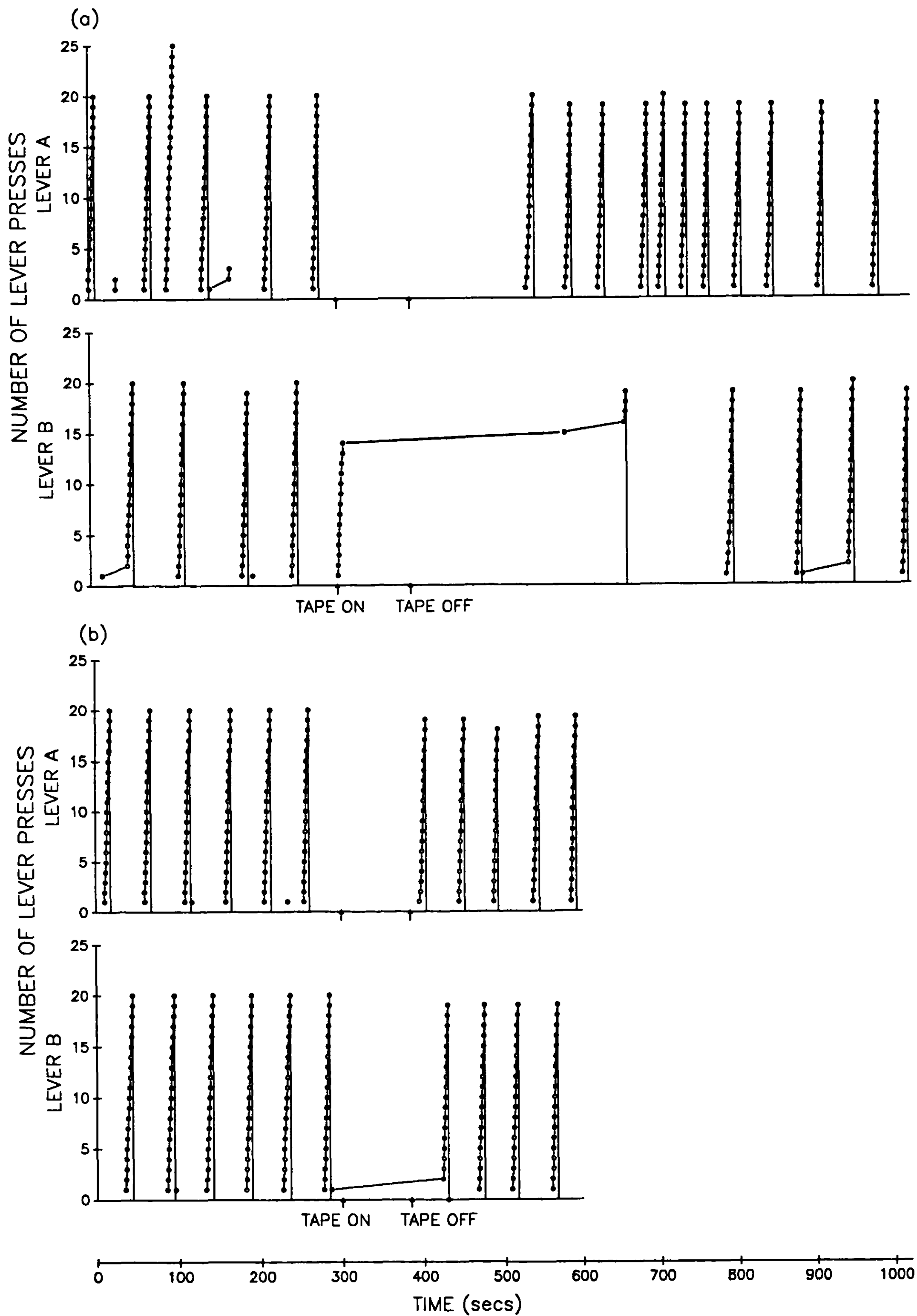


Fig. 7.9 Cumulative records of the discriminatory response of pigs presented with a tape recording of a barking dog of duration 89 secs starting 300 secs after a response to a saline pretreatment: (a) response of pig C1 (PTZ lever=A), (b) response of pig C4 (PTZ lever=B).

The availability of food

Intensive animal husbandry involves keeping large numbers of conspecifics in an environment which imposes several restrictions on the animals such as restricted food availability. Such restrictions may result in frustration and this state has been implicated as an important factor in the development of abnormal physiological and behavioural reactions observed in farm animals (Wood-Gush et al., 1975). In a previous study, it has been demonstrated that pigs participate in displacement behaviour and have increased plasma corticosteroid levels when an operant procedure, normally associated with food reinforcement is conducted in extinction (Dantzer et al., 1980). The aim of the present study was to examine the psychological state of pigs subjected to frustrated non-reward. An attempt was made to induce frustration in pigs C1, C3, C4 and C5 by restricting food availability during an operant session in the Skinner box. Food availability was restricted by a psychological obstacle rather than the usual physical obstacle of withholding food reward, as employed by Dantzer et al. (1980). This approach was adopted to avoid any interference with the PTZ/saline discrimination performance of these animals.

An attempt was made to introduce an aspect of novelty to the food bowl of the Skinner box since previous observations showed that pigs were reluctant to eat from a novel food container, despite being very hungry. To this end, a small rubber ball was placed in the food bowl and then the animals were tested. The results of tests conducted with pigs C3 and C5 are shown in Fig. 7.10. The drug designated lever was predominantly selected by pig C3 until the ball was removed from the food bowl at 433.3 seconds after the start of the test. Following this event, the animal selected both levers alternately. This pig did not begin to consume the food reinforcements obtained from each lever selection until 145 secs following the start of the test. The drug designated lever was also predominantly selected by pig C5 and during the test period only three selections were made on the alternate lever. At 422 secs after the start of the test, the animal turned around in the box to face the exit and the test was terminated. This pig did not eat any of the food reinforcements obtained during the session. The presence of the red ball in the food bowl also disrupted the alternation of lever selection response of pig C1 whereby 3

consecutive selections were initially made on the B lever (non-drug lever; result not shown). In contrast, the alternation of lever selection response of pig C4 was not disturbed by the ball in the food bowl (result not shown) and this animal removed the object from the bowl after the first two selections. Following each lever selection pigs C1 and C4 consumed the resultant food reinforcement.

The consumption of food reinforcements by pigs C3 and C5 was obstructed by the introduction of a novel stimulus to the food bowl. Since these animals displayed an anxiogenic response to the psychological obstacle, the present results show that frustration induced by restricted food availability corresponds to a state of anxiety.

The introduction of novel stimuli

A consequence of the environmental restrictions imposed by intensive animal husbandry systems is that animals are periodically exposed to completely novel environments. Some of the novel environments to which pigs are typically exposed have already been discussed above, e.g. transportation. However, since specific aspects of such environments, such as novelty, were not studied, the present investigation was designed to examine the effect of a specific novel stimulus on the psychological state of the animal.

The wire mesh floor of the Skinner box was covered with an even sheet of smooth surfaced wood and the pigs were then tested. An anxiogenic response to this stimulus was observed in three out of four pigs. These animals selected the drug lever only, during the initial period of the test session. The period of response exclusive to the drug lever was 209.9 secs for pig C5 (Fig. 7.11a), 58.9 secs for pig C4 (Fig. 7.11b) and 90.6 secs for pig C1 (result not shown). The animals selected both levers alternately for the remainder of the test period. The novel floor did not affect the alternation of lever selection response of pig C3 (result not shown).

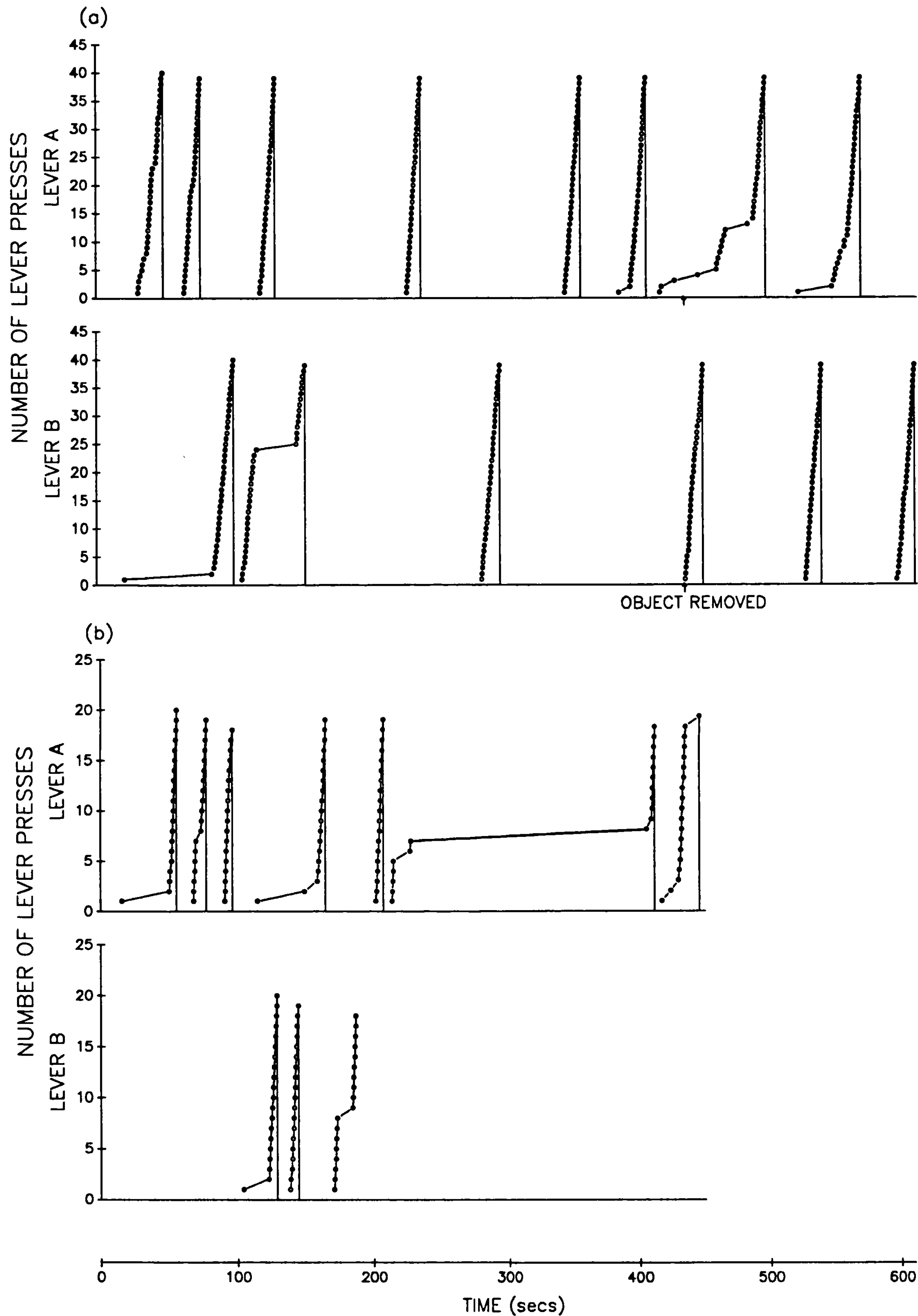


Fig. 7.10 Cumulative records of the effect of restricted food availability on the discriminatory response of pigs: response of pig C3 (a; PTZ lever=A) and response of pig C5 (b; PTZ lever=A), to the presence of a novel object (rubber ball) in the food bowl.

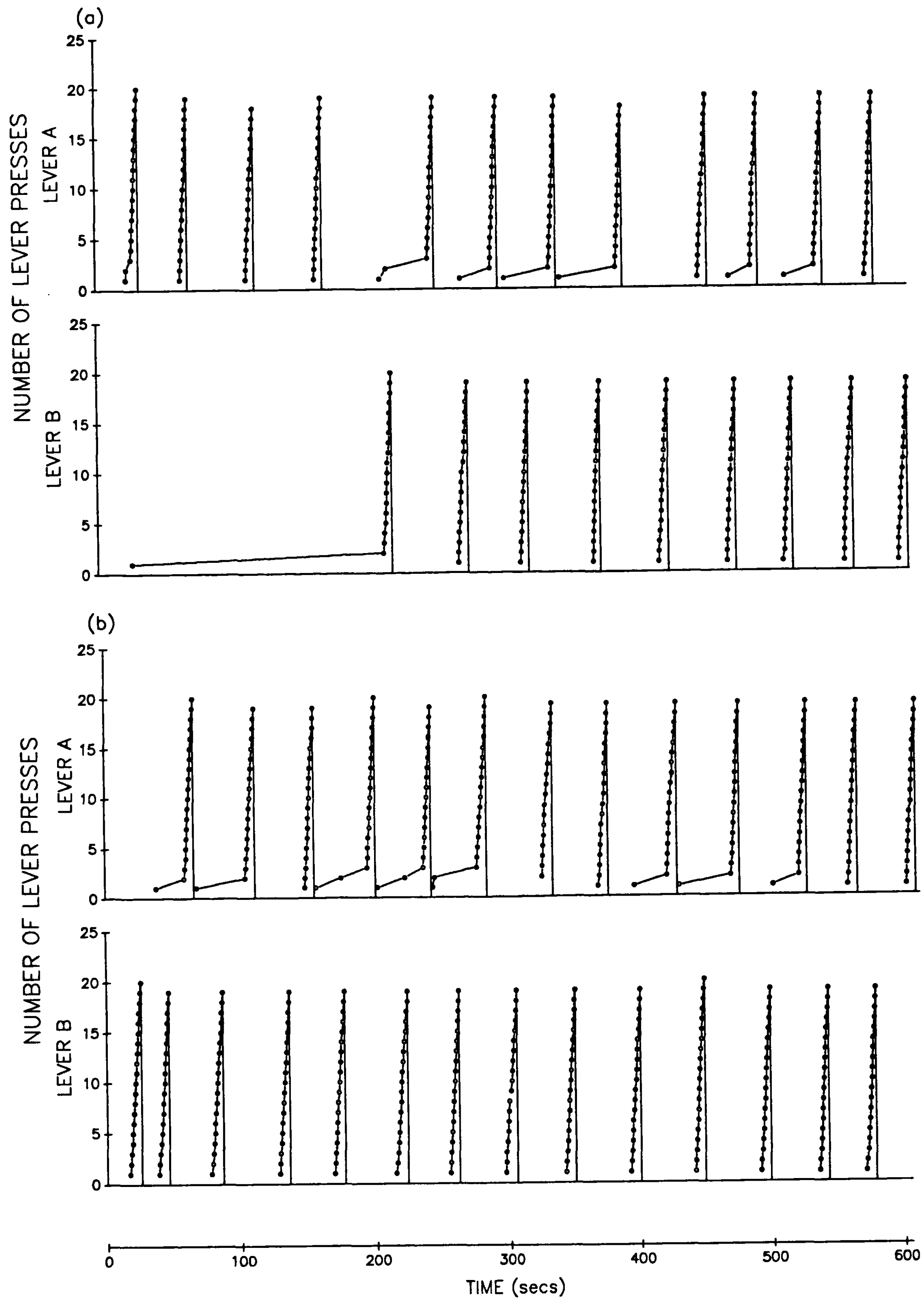


Fig. 7.11 Cumulative records of the effect of a novel floor in the Skinner box on the discriminatory response of pigs: response of pig C5 (a; PTZ lever=A) and response of pig C4 (b; PTZ lever=B), in the presence of a sheet of wood covering the wire mesh floor of the test box.

The results described above, demonstrate an anxiogenic response of pigs to stimuli which mimic certain variables encountered in pig husbandry. The observed anxiogenic responses to such stimuli (e.g. change of temperature, abattoir condition, introduction of novelty) were typically short lasting and the animals usually showed habituation to the anxiogenic properties of these aversive stimuli as revealed by an alternation of lever selection response. However, certain stimuli produced intense anxiety or a suppression of operant behaviour. For instance, the playback of a recording of a squealing handled pig to pig C1 resulted in response suppression (Fig. 7.8.a) while frustrated non-reward induced an anxiogenic response in pig C5 (Fig. 7.10.b) and in both cases the tests were terminated because the animal stopped responding and faced the exit of the box. It was, therefore, not possible to monitor a recovery of an alternation of lever selection response. The failure of pigs C1 and C5 to habituate to these stimuli probably reflects a continued sense of threat and the non-removal of the obstacle preventing consummatory behaviour, respectively.

Of the environmental stimuli examined in this study, 7/12 were shown to induce an anxiogenic response in pig C1, 3/8 in pig C3, 3/12 in pig C4 and 3/12 in pig C5, indicating individual susceptibility to anxiety. Although none of the stimuli tested produced an anxiogenic response in all of the pigs, the present evaluation probably underestimates the anxiogenic effects of such stimuli in pig husbandry since the pigs used in this study were not subject to the conditions typical of intensive husbandry systems. Also, in contrast to animals kept in such conditions, the pigs employed in the present experiments had already experienced several novel environments e.g. transportation from the breeding source to the college, the laboratory and conditioning room. Such differences in housing conditions and past experience of various stimuli might also account for the failure of the present study to detect an anxiogenic response to changes in ambient lighting conditions. The effect of a dramatic change from light to darkness or vice versa on the discriminatory response of pigs was examined by turning on or off the lights in the conditioning room while the animals were responding to a saline pretreatment. In two animals, such changes in ambient lighting had no effect on the alternation of lever selection response while a third animal showed an anxiogenic response when

the test was initiated under darkened conditions (results not shown). Nevertheless, since the various stimuli tested typically induced an anxiogenic response in two out of four animals, the present study suggests that certain husbandry situations should be avoided or modified, in order to prevent unnecessary psychological distress in these animals. For instance, the handling or treatment of pigs causing intense vocalisation reactions could be conducted in isolation from other animals.

A more precise evaluation of the anxiogenic nature of certain variables of pig husbandry would benefit from a modification of the present test procedure, such that the response of the animal could be monitored during the putative aversive event. For variables, such as transportation, a simulation of the event such as that conducted by Stephens and coworkers (1985) would enable a distinction of the various anxiogenic components of the process.

7.3 Conclusions

Environmental stimuli which mimic certain variables encountered in pig husbandry systems were shown to induce a psychological state in pigs which generalised to the PTZ cue. The detection of anxiety induced by stimuli representative of variables in pig husbandry, such as the mixing of strange pigs or the housing of these animals in restricted environments, may explain the aberrant behaviour which is known to occur in these situations. The results of the present study suggests that certain variables of pig husbandry systems should be avoided or modified to prevent the occurrence of unnecessary psychological distress in these animals. The present evaluation demonstrates that pigs rapidly habituate to many aversive stimuli such as novelty or olfactory cues associated with the presence of strange pigs or abattoir conditions. It is possible, therefore, that a gradual introduction of such stimuli and the subsequent habituation to these stimuli, may reduce the distress experienced at the time of the actual event. Further evidence of the anxiogenic properties of certain pig husbandry variables could be obtained by a modification of the test procedure currently employed, such that the response of the animals could be assessed at the time of occurrence of the putative aversive event.

The ability of the PTZ discrimination paradigm in pigs to detect anxiety induced by stimuli normally present in the environment of these animals, demonstrates the validity of this test as a model of anxiety in the pig. The findings reported in this chapter corroborate the results obtained from the behavioural evaluation of the PTZ cue (Chapter 6) which revealed that the PTZ cue corresponds to a state of anxiety rather than a preconvulsant aura. In agreement with these findings, two studies of the anxiogenic nature of the PTZ and FG7142 cue in rats have revealed a generalisation of the state induced by the experience of a home intruder or a novel environment,respectively, to the discriminative drug stimulus (Vellucci et al., 1988; Leidenheimer & Schechter, 1988).

Chapter 8
Conclusions

An attempt was made to study the influence of ovarian steroids and environmental stimuli on the state of anxiety in the mouse and pig respectively. In Chapter 1, an assessment of some of the available animal models of anxiety revealed that the PTZ discrimination paradigm was devoid of many of the limitations imposed by other tests. However, since this model requires animal training it was inappropriate for the study of the effect of ovarian cycle stage on the anxiety state. For such a study, an animal model was necessary where naive animals could be employed and thereby results could be interpreted free from ambiguities of state dependent learning. Hence, a model of exploration, the light/dark choice test was applied to this study.

Previous reports had revealed that the light/dark box test was sensitive to the detection of anxiolytic drug activity (Crawley, 1981) and to changes in exploratory behaviour during the mouse oestrous cycle (Gray, 1978). In Chapter 3, the behaviour of untreated and diazepam treated male and female mice tested in the light/dark choice apparatus is described. Contrary to the finding of Gray (1978), changes were not observed in the behaviour of untreated female mice during the oestrous cycle. In agreement with a previous report (Crawley, 1981), diazepam induced a dose-related increase in the proposed index of anxiety, the number of light/dark transitions, in male mice. However, in female mice fluctuations in sensitivity to this action of diazepam were observed; at the test dose used only females at the oestrus and dioestrus cycle stages showed a diazepam induced increase in transitions, while female mice at pro-oestrus, metoestrus II and late dioestrus showed no response. Furthermore, a paradoxical effect was observed in mice at metoestrus I in that the test dose of diazepam induced a decrease in the number of light/dark transitions. According to other authors (Crawley, 1981; Crawley et al., 1984; Belzung et al., 1987) this effect would be interpreted as an anxiogenic drug action. Additional experiments revealed that this effect was not due to a change in the metabolism or distribution of diazepam or to an enhanced sensitivity to a sedative action of the drug at this stage of the cycle.

Since the proposed conflict of the test is the tendency to explore a brightly lit chamber versus the tendency to retreat into a dark chamber, other parameters of the behaviour of mice tested in the apparatus which should indicate a shift in such a conflict were monitored. This analysis produced ambiguous results. While diazepam increased the number of light/dark transitions made by male mice and female mice at oestrus and dioestrus, other parameters which would indicate decreased light aversion such as the latency to emerge from dark to light chamber or the time in the light chamber did not change in response to the drug. In contrast, the diazepam induced decrease in transitions at metoestrus I was accompanied by a change in these parameters indicative of increased light aversion.

In an attempt to explain the above results, an evaluation of the light/dark box test was conducted. The results from this evaluation are presented in Chapter 4 and reveal that a diazepam induced increase in light/dark transitions reflects a stimulant action of the drug and such a behavioural change is not specific to diazepam since the same effect was achieved with the psychomotor stimulant d-amphetamine. In addition, the results presented in Chapter 4 demonstrate that a change in the light/dark transitions parameter is not dependent on a light/dark conflict and that increased exploration of the light chamber becomes evident in untreated mice only when locomotor behaviour is reduced by repeated exposure of the animals to the apparatus. Hence, this test is inappropriate for a study of anxiety in naive animals or in response to a drug treatment such as diazepam which produces a stimulant effect. However, results obtained from tests conducted with d-amphetamine indicate that the test maybe sensitive to an anxiogenic drug action in naive animals.

On the basis of the above evaluation, some interpretations may be made of the results obtained from the use of the test in the experiments described in Chapter 3. (1) The inability to detect changes in exploratory behaviour of untreated female mice during the oestrous cycle may reflect the insensitivity of the test to decreased light aversion in naive animals and therefore the present study does not account for possible anxiolytic effects of endogenous

steroids and their metabolites in untreated animals. (2) The diazepam induced increase in transitions in female mice at oestrus and dioestrus reflects a stimulant action of the drug. (3) The effects of diazepam on female mice at metoestrus I would appear to represent an anxiogenic drug action since three parameters of the behaviour of these mice showed a drug induced shift in favour of increased light aversion. Although, these changes were accompanied by a decrease in light/dark transitions, the drug induced change in the latter parameter may not be related to a change in exploratory behaviour. This assumption is made since experiments with d-amphetamine and test habituated untreated animals revealed that indices of exploratory behaviour decrease or increase respectively independent of a similar direction of change in the number of light/dark transitions (Chapter 4). This last conclusion would benefit from a corroboration from further investigations such as an evaluation of the behaviour of male mice tested in the light/dark box after treatment with a specific anxiogenic drug or exposure to the home cage of a strange mouse. Such a 'home-intruder' experience has been shown to produce an anxiogenic effect in the rat (Vellucci et al., 1988).

The results obtained from the evaluation of the light/dark box (Chapter 4) support the criticisms made in Chapter 1 of tests whose indices of anxiety rely on locomotor performance and demonstrate the need for measures of task specific exploration and behavioural evaluations before such animal models are used in studies of anxiety.

Several physiological mechanisms were suggested to account for the fluctuations in sensitivity to diazepam during the oestrous cycle (Chapter 3). The most likely explanation for these observations would be a genomic action of progesterone regulating either the function of its own metabolites which have known direct effects on GABA synaptic function (see Chapter 1, Section 1.3.b) or the expression of the $GABA_A$ receptor complex subunits or the GABA synthetic enzyme glutamate decarboxylase. This explanation was put forward because the two oestrous cycle stages, oestrus and dioestrus at which sensitivity to diazepam was highest are the only stages preceded by an increased production of progesterone (Butcher et al., 1974; Nelson et al., 1981).

Such steroid regulated changes in GABA synaptic function may account for the changes in mood, seizure frequency or consumption of minor tranquillisers known to occur during the menstrual cycle. In addition, the apparent anxiogenic action of diazepam at metoestrus I may have serious implications when considering the anxiety state reported by many women during the premenstrual phase and its treatment with benzodiazepine tranquillisers.

To study anxiogenic aspects of pig welfare, an attempt was made to establish the PTZ discrimination paradigm in the pig. Initially pigs were pharmacologically conditioned according to the procedure previously established for rats (Shearman & Lal, 1979). As reported in Chapter 5, pigs learned to discriminate the effects of PTZ. However, as indicated from studies with rats (see Lal & Emmett-Oglesby, 1983), the conditioning procedure was subject to a major limitation in that only the response of the animal prior to the first lever reinforcement could be accepted as a valid index of anxiety. Since the aim of the present study was to apply the PTZ conditioning paradigm to the evaluation of pig welfare, with such a response time limitation, this study would not be feasible. In addition, prior to such an application of the test, the present study proposed to conduct a behavioural evaluation of the anxiogenic nature of the PTZ cue.

To facilitate the above studies a novel pharmacological conditioning procedure was developed in the pig. As described in Chapter 5, this procedure enabled the detection of the onset and the offset of the PTZ stimulus within a single test session. In Chapter 6, evidence is presented which shows that the state induced by a conditioned anxiogenic stimulus generalised to the PTZ cue in a diazepam sensitive manner. In addition, an experiment is also described which reveals that the PTZ cue and a conditioned emotional state originate through a common mechanism. These findings provide firm evidence that the PTZ cue in pigs relates to a state of anxiety. In agreement with other studies of the PTZ cue in the rat (Stephens et al., 1984), a pharmacological evaluation revealed that the PTZ cue in pigs was sensitive to specific antagonism by diazepam. Such an antagonism was not observed after pretreatment with the

anticonvulsant agent ethosuximide. An indication of an anxiogenic action of the ß-carboline FG7142 is also reported.

In Chapter 7, results obtained from the application of the PTZ discrimination paradigm to the evaluation of anxiogenic aspects of pig welfare, are described. These data reveal that the test is sensitive to the detection of anxiety induced by stimuli normally encountered in pig husbandry.

The evidence provided in this thesis shows that the PTZ discrimination paradigm in pigs is a valid test of anxiety. In addition to its potential usefulness in the investigation of pig welfare, it provides a useful psychopharmacological tool for studies of the neurobiology and physiology of anxiety. As mentioned at the beginning of the present Chapter, this procedure is not appropriate for studies such as the determination of ovarian cycle influences on the anxiety state of the intact animal. However, the PTZ discrimination paradigm could be applied to the investigation of steroid effects on anxiety by monitoring the discriminatory behaviour of ovariectomized animals treated with various steroids or their metabolites. Nevertheless, as discussed in Chapter 3 (Section 3.1) the validity of such investigations is dependent on corroborative evidence obtained from evaluations of the effects of steroids on anxiety in the intact animal. This emphasises the need for a valid model of anxiety based on unconditioned behaviour. The results presented in this thesis demonstrate that the light/dark box test of exploration is not sensitive to anxiolytic activity in naive animals and therefore in hindsight the elevated plus-maze or the holeboard test may have been a better choice for the study conducted on the mouse oestrous cycle. However, the light/dark box test did provide a measure of diazepam sensitivity and since this sensitivity was observed to fluctuate during the oestrous cycle, the present study demonstrates a modulatory role of ovarian steroids on GABAergic function.

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