

PROTEIN SYNTHESIS INHIBITION AND SHORT AND LONG TERM  
HABITUATION OF THE DORSAL ANTENNAE WITHDRAWAL RESPONSE  
IN HELIX ASPERSA

BY

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## ABSTRACT

### PROTEIN SYNTHESIS INHIBITION AND SHORT AND LONG TERM HABITUATION OF THE DORSAL ANTENNAE WITHDRAWAL RESPONSE IN HELIX ASPERSA, BY S. RAY

The pharmacological disruption of memory by various protein synthesis inhibitory drugs (PSIs) has implicated protein synthesis as a requirement for long term learning but not for short. However, evidence derived from PSI research remains equivocal, with the apparent amnesic effects of PSIs being attributed to drug side-effects and general behavioural debilitations.

Research reported in this thesis investigates the behavioural effects on short and long term habituation of the dorsal antennae withdrawal reflex in the snail (Helix aspersa) of three antibiotic drugs known to reversibly inhibit protein synthesis; anisomycin, actinomycin D, and puromycin. Initially, habituation was established as true learning in the snail and was demonstrated to be capable of retention for over 24 hours from one training session and over 6 months from a series of training sessions. The parametric characteristics of both short and long term habituation in the snail was established and found to be identical to those demonstrated in vertebrate habituation. Such characteristics were found to be different for short and long term habituation. Injection of PSIs showed no effect on short term habituation but disrupted long term habituation if PSI was active within a 'critical time window' during or for approximately 40 minutes after training. Later injections had no amnesic effect, and neither did injections 2 hours prior to training. The amnesic effects were demonstrated not to be attributable to drug side-effects by the development and application of a 'behavioural test battery' to screen general snail behaviour for drug induced debilitations at a variety of doses. Dose/amnesic effect relationships are also reported. Potentially confounding effects, such as, state dependent learning, and drug performance effects, were controlled out. The effects of the PSIs on short and long term habituation are then reported in terms of their effects on the established short and long term parametric characteristics of the learning. Drug injected snails showed normal short term parametric characteristics in training. However, in a long term retest drug treated snails also showed the parametric characteristics of short term habituation which demonstrated the degree of induced amnesia.

The results are discussed in terms of a gene expression model of long term habituation and suggest that short and long term habituation are mediated by different processes. Short term habituation is protein synthesis independent and long term habituation is protein synthesis dependent.

## DECLARATIONS

- 1) While registered for this degree, I have not been a registered candidate for another award of the C.N.A.A. or of a University.
- 2) None of the material contained herein has been used in any submission for an academic award.

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Research reported in this thesis investigates the behavioural effects on short and long term habituation of the dorsal antennae withdrawal reflex in the snail (Helix aspersa) of three antibiotic drugs known to reversibly inhibit protein synthesis; anisomycin, actinomycin D, and puromycin. Initially, habituation was established as true learning in the snail and was demonstrated to be capable of retention for over 24 hours from one training session and over 6 months from a series of training sessions. The parametric characteristics of both short and long term habituation in the snail was established and found to be identical to those demonstrated in vertebrate habituation. Such characteristics were found to be different for short and long term habituation. Injection of PSIs showed no effect on short term habituation but disrupted long term habituation if PSI was active within a 'critical time window' during or for approximately 40 minutes after training. Later injections had no amnesic effect, and neither did injections 2 hours prior to training. The amnesic effects were demonstrated not to be attributable to drug side-effects by the development and application of a 'behavioural test battery' to screen general snail behaviour for drug induced debilitations at a variety of doses. Dose/amnesic effect relationships are also reported. Potentially confounding effects, such as, state dependent learning, and drug performance effects, were controlled out. The effects of the PSIs on short and long term habituation are then reported in terms of their effects on the established short and long term parametric characteristics of the learning. Drug injected snails showed normal short term parametric characteristics in training. However, in a long term retest drug treated snails also showed the parametric characteristics of short term habituation which demonstrated the degree of induced amnesia.

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## CONTENTS

### PROTEIN SYNTHESIS INHIBITION AND SHORT AND LONG TERM HABITUATION OF THE DORSAL ANTENNAE WITHDRAWAL IN HELIX ASPERSA

#### CHAPTER 1

##### INTRODUCTION

Protein synthesis and learning .....	Page 1.
1.2. Protein synthesis inhibition and .....	Page 19.
Habituation	
1.3. Gene expression and learning .....	Page 20.
1.4. Aims of Thesis .....	Page 24.

#### CHAPTER 2

##### HABITUATION

Habituation ....	Page 27.
2.1 Evidence for long term habituation .....	Page 29.
2.2 Long term habituation in invertebrates ....	Page 32.
2.3 The Differentiation of short and long ....	Page 33.
term habituation	
2.3.1 Stimulus intensity effects and short ...	Page 36.
and long term habituation	

2.3.2 The effects of massed and spaced .....	Page 38.
training on short and long term habituation	
2.3.3 Interstimulus interval effects .....	Page 39.
2.3.4 Evidence from other forms of .....	Page 50.
massed and spaced training	
Comment .....	Page 57.

### CHAPTER 3

#### ESTABLISHMENT OF A SUITABLE LEARNING BEHAVIOUR

Habituation in the snail .....	Page 59.
Experiment 1 .....	Page 63.
Experiment 2 .....	Page 68.
Experiment 3 .....	Page 70.
Experiment 4a .....	Page 74.
Experiment 4b .....	Page 77.
Experiment 4c .....	Page 81.
Experiment 4d .....	Page 83.
Experiment 4e .....	Page 85.
General Discussion .....	Page 88.

### Chapter 4

#### LONG TERM HABITUATION IN THE SNAIL HELIX ASPERSA

Retention and recovery of response .....	Page 90.
from one training session	
Experiment 1 .....	Page 91.

Experiment 2 .....	Page 94.
General Discussion .....	Page 97.
4.2. The effects of multiple training .....	Page 99.
sessions on long term habituation	
Experiment 3 .....	Page 99.
Experiment 4 .....	Page 103
Experiment 5 .....	Page 107
Experiment 6 .....	Page 110
General Discussion .....	Page 113

## Chapter 5

### THE EFFECTS OF INTERSTIMULUS INTERVAL ON SHORT AND LONG TERM HABITUATION

Introduction .....	Page 116
5.1. The effects of ISI on short term .....	Page 117
habituation	
Experiment 1 .....	Page 117
Experiment 2 .....	Page 119
5.2. ISI effects on long term habituation .....	Page 123
Experiment 3 .....	Page 123
Experiment 3b .....	Page 127
Experiment 4 .....	Page 130
Experiment 5 .....	Page 131
General Discussion .....	Page 134



## Chapter 6

### THE EFFECTS OF MASSED AND SPACED TRAINING ON SHORT AND LONG TERM HABITUATION

Introduction .....	Page 137
Experiment 1 .....	Page 138
Experiment 2 .....	Page 142
Experiment 3 .....	Page 146
General Discussion .....	Page 151

## Chapter 7

### PROTEIN SYNTHESIS INHIBITORS, SNAILS AND INJECTION METHODOLOGY

Introduction .....	Page 156
7.1 The Drugs .....	Page 158
7.2 Injection techniques and site of action .....	Page 158
7.3 Dose of Drugs employed .....	Page 160
7.4 Purity of Drugs .....	Page 161
7.5 Uptake of Drugs and inhibition of .....	Page 162
protein synthesis in the snail	
Results .....	Page 163

## Chapter 8

### THE DEVELOPMENT OF A BATTERY OF BEHAVIOURAL TESTS TO SCREEN ANTIBIOTICS FOR GENERAL EFFECTS ON SNAIL BEHAVIOUR

Introduction .....	Page 166
8.1 Open field test .....	Page 169
8.2 Withdrawal reflex tests .....	Page 170
8.3 Activity level .....	Page 171
8.4 Detection of hidden food source .....	Page 172
8.5 Feeding behaviour .....	Page 173
8.6 Righting Reflex .....	Page 173
Experiment 1 .....	Page 175
Experiment 2 .....	Page 179
Experiment 3 .....	Page 183
Experiment 4 .....	Page 185
Experiment 5 .....	Page 187
Experiment 6 .....	Page 189
General Discussion .....	Page 191

## Chapter 9

### DOSE EFFECT ANALYSIS OF THE ANTIBIOTICS- ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN ON SHORT AND LONG TERM HABITUATION

Introduction .....	Page 193
Method .....	Page 194

Results .....	Page 196
Discussion .....	Page 197

Chapter 10

THE EFFECTS OF PSIS ON SHORT AND LONG TERM  
HABITUATION OF THE ANTENNAE WITHDRAWAL RESPONSE

Experiment 1 .....	Page 199
Experiment 2 .....	Page 209
Experiment 3 .....	Page 214
General Discussion .....	Page 219

Chapter 11

THE EFFECTS OF PSIS ON THE DEVELOPMENT OF SHORT & LONG TERM  
HABITUATION AND ITS RETENTION WITH A FIXED TRIALS  
TRAINING TRIALS

Introduction .....	Page 222
Method .....	Page 223
Results .....	Page 225
Discussion .....	Page 230

## Chapter 12

### STRESS: AN ALTERNATIVE EXPLANATION OF THE EFFECTS OF PSI ON SHORT AND LONG TERM HABITUATION

Experiment 1 .....	Page 232
Experiment 2 .....	Page 240
General Discussion .....	Page 245

## Chapter 13

### A CRITICAL TIME WINDOW OF AMNESIC EFFECTS OF ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN AND THE EFFECTS OF DOSE ON SUCH TIME/EFFECT RELATIONSHIPS

Introduction and general methodology .....	Page 247
Experiment 1 .....	Page 253
Experiment 2 .....	Page 258
Experiment 3 .....	Page 263
General Discussion .....	Page 268

## Chapter 14

### SHORT TERM HABITUATION AND PROTEIN SYNTHESIS INHIBITION

Introduction .....	Page 272
Method .....	Page 275
Results and Discussion .....	Page 276

## Chapter 15

### EVIDENCE OF SOME RETENTION FROM SHORT TERM HABITUATION IN THE ABSENCE OF PROTEIN SYNTHESIS

Introduction .....	Page 278
Experiment 1 .....	Page 280
Experiment 2 .....	Page 283
General Discussion .....	Page 287

## Chapter 16

### EXTENT OF AMNESIC EFFECT IN LONG TERM FOLLOWING PROTEIN SYNTHESIS

Introduction .....	Page 289
General method .....	Page 290
Experiment 1 .....	Page 291
Experiment 2 .....	Page 292
Experiment 3 .....	Page 294
General Discussion .....	Page 295

## Chapter 17

### PROTEIN SYNTHESIS INHIBITION AND MULTIPLE TRAINING SESSION EFFECTS

Introduction and General methodology .....	Page 297
Experiment 1 .....	Page 301

Experiment 2 .....	Page 304
Experiment 3 .....	Page 307
Experiment 4 .....	Page 309
General Discussion.....	Page 312

## Chapter 18

### GENERAL DISCUSSION

Discussion .....	Page 316
18.1 Short term habituation.....	Page 323
18.2 Long term habituation .....	Page 326
18.3 Stages of memory formation and.....	Page 330
protein synthesis inhibition	
18.4 Speculations on mechanisms of habituation ..	Page 335
18.5 Summary and Conclusions .....	Page 347
REFERENCES .....	Page 348

## INTRODUCTION

One of the major questions addressed by physiological psychology is the biological basis of learning and memory. One aspect of this which has attracted a considerable focus of attention over the past thirty years is the possible involvement of proteins and their synthesis in such a process.

Interest in protein synthesis and memory was initiated by Katz and Halstead (1950) who hypothesised a memory trace that involved protein molecules. Since then, interest in protein synthesis and memory has generated a prolific literature with protein involvement suggested by a variety of experimental approaches. By far the most work has been carried out on the effects of pharmacological inhibition of protein synthesis by antibiotic agents protein synthesis inhibitors, (PSIs). However, before commencing an investigation of the PSI technique, evidence of protein involvement from other sources must be mentioned.

Interventive techniques such as PSIs are not the only source of evidence implicating protein synthesis in the biological mechanism of learning.

One of the major sources of evidence implicating proteins has been research involving various labelling techniques. Early research in this area has been criticised by some authors for its lack of specificity of the biochemical correlates implicated and poor reporting of control group data (e.g. Brown & Harding, 1969); and Dunn, 1976a). While more contemporary research has striven to

overcome these problems of specificity and control, some of the results are conflicting concerning the specificity of this action. For example Yanagihara & Hyden (1971) reported a significant increase in labelled proteins in pyramidal neurons CA1 and CA3 but not CA4 after 5 days of training. This was with [3H]leucine incorporation on a reversal of handedness task in the rat. However, the same report also showed that TCA-soluble 3H was not changed. The relative specific activities (RSA's) of the proteins (protein 3H divided by TCA-soluble 3H) were increased in cells CA3 and CA4, but not CA1. This study, like many others, did conclude that there was an increase in protein synthesis with learning by measuring incorporation of a radioactive label.

Other evidence, from a variety of learning behaviours and biochemical and immunochemical techniques, would support such a conclusion. Shashoua, in a series of studies, reported changes in RNA metabolism in the brains of goldfish after learning to adopt a normal swimming position with a large plastic float attached to their ventral surface (Benowitz & Shashoua, 1977; Shashoua, 1970, 1976a,b, 1977b). These researchers went on to report the effects of specific antisera to specific proteins which increased with the behavioural task and reported that they had specific amnesic properties (Shashoua, 1977b; Shashoua & Moore, 1978). They have extended their research to include immunohistochemical analysis and reached the same conclusions with regard to antisera (Benowitz & Shashoua, 1977; Shashoua, 1979). This research has been regarded by some researchers as problematic both behaviourally and procedurally, and its



cross species relevance is questioned because of dissimilar results in the chick brain (Benowitz & Shashoua, 1979). The studies of Shashoua remain one of the few that have used interventive approaches (e.g. antisera experiments with correlational approaches such as labelling).

Hyden & Lang (1975) showed regional and, to some extent, molecular specificity in an increase in incorporation following a re-reversal runway task to light cue and water reward. Using a brightness discrimination task, Matthies and colleagues showed increases of protein and glycoprotein incorporation (Pohle, Lossner, Ruthrich, & Mathis, 1974; Pohle & Matthias, 1971, 1974, 1976). Using electrophoresis to separate labelled proteins, suggestions of increased specific incorporation are supported by Popov et al. (1975a,b; 1976b).

Labelling research has also been carried out on the visual system of the rat (Krivaneck, 1974; 1975), and the spinal cord of the frog during shock avoidance conditioning (Peters, 1976; Peters & Wirth, 1976). Passive avoidance learning paradigms have not escaped the labelist either (e.g. Glushchenko, Pevsner, & Brumberg, 1977). Again, with passive avoidance, an increase in protein synthesis is associated with the learning. Passive avoidance paradigms have also served to address a possible confound of this approach; namely, precursor pool changes. Hershkowitz, Wilson, & Glassman (1975) used a variety of labelled precursors to negate the problem and report comparative evidence from these different labels which show the increased labelling could not be attributed to depletion of

precursor pool. The above research suggests changes in the labelling of cerebral, free, amino acids following peripheral injections, and similar results have been achieved in cerebral incorporation studies (Dunn et al., 1978; Hambley et al., 1977; Hershkowitz et al., 1975; Hershkowitz & Wilson 1976).

Perhaps the most significant development of this approach has occurred with studies which have shown increased incorporation of fucose into fucosyl glycoproteins during learning (Barraco & Irwin, 1976; Bogoch, 1968; Chapouthier, 1977; Damstra-Entingh, Entingh, Wilson, & Glassman, 1974; Damstra, Entingh, & Wilson, 1975; Haywood, Hambley, & Rose, 1975a; Irwin & Samson, 1971; Irwin, & Terrian, 1978; Rose, 1986; Routtenberg, George, & Davis, 1974; Savaki & Lewis, 1977; Zomley-Neurath & Keller, 1977).

Hambley & Rose (1977) have demonstrated extremely complex changes in the cyclic AMP content and in adenylate cyclase activity in areas of the precocial brain after imprinting. Biochemical changes associated with imprinting are summarised by Rose, Gibbs, & Hambley (1979)<sup>who</sup> measured the muscarinic acetylcholine receptors by in-vitro binding of [3H]quinuclidinyl benzilate (QNB) in extracts of chick forebrain and optic lobes after passive avoidance training. QNB-binding was increased 10 and 30 min but not 180 min after training in the forebrain of chicks trained with methlanthranilate as opposed to water. No change was observed in the optic lobes at any time. Acetylcholinesterase activity was not altered by training

(as opposed to Haywood et al. 1975a, who reported an increase after imprinting). Interestingly, when the chicks were treated with cycloheximide or ouabain, 5 minutes before training, QNB binding changes did occur 30 min after training. Ouabain treated chicks were amnesic when tested 30 minutes after training although cycloheximide treated chicks were not. Earlier data have shown that cycloheximide induced amnesia develops more slowly (Gibbs & Ng, 1977). An increase in tubulin content of the chick in the same region of the brain during the same behaviour has also been reported (Mileusnic, Sukumar, & Rose, 1979). Although the interpretation of binding data is to some extent problematic (e.g. conformational changes or changes in the occupancy of binding sites), these problems are different from those encountered by in-vivo labeling. Together the two approaches do suggest a metabolic activation of cells in the anterior forebrain roof of chicks during passive avoidance behaviour.

Further to the above evidence, Burgoyne & Rose (1980) report evidence that changes in glycoproteins are not very fast. They examined the incorporation of radioactive lysine following the exposure of dark reared rats to light and found an increase within one hour after the beginning of the exposure. Radioactive fucose was incorporated into the glycoprotein sugar moiety after about three hours when the lysine incorporation was already depressed. In one day old chicks, passive avoidance training increased the incorporation of radioactive fucose into glycoproteins of the synaptic membranes, and all nine peaks of glycoprotein were labelled (Burgoyne & Rose, 1980a).

Evidence from the above studies using a variety of labelling techniques, learning behaviours, and species leaves little doubt that the brain is altered metabolically when learning occurs. The above evidence implicates proteins or glycoproteins in this process. Recent research has provided valuable evidence on the nature of synaptic glycoprotein alteration during learning in the chick (Rose, 1986). The availability, and increased use of molecular biological techniques in neuroscience makes the above evidence of critical importance to our understanding of the role of protein synthesis in learning. One problem, however, is the rarity with which interventive techniques and correlative techniques are used in the same laboratory, or even comparisons between interventive techniques, such as antibiotics and labelling techniques, are made. The labelling approach can yield vital data on how and where new proteins are made and used in learning. However, such research would benefit from a solution to the long standing problems of the main interventive approach to studying protein synthesis and learning namely antibiotic effects on short and long term memory.

While the above evidence implicates new protein synthesis in learning, the main evidence for protein involvement is derived from studies using PSIs. This approach, despite a considerable volume of research remains problematic and has attracted many criticisms. This thesis is addressed to these problems. Below is a review of research employing PSIs which serves to outline some of these problems to be addressed. Overcoming these problems

would serve to enhance evidence from the other methodological approaches mentioned above and broach the broader issue of genomic involvement in learning and memory.

As stated above, the focus of this thesis is on the technique which provided the first empirical support for Katz and Halstead's theory; the pharmacological inhibition of protein synthesis and its effect on learning and memory. Initial support for their theory was provided over ten years later by Flexner, Flexner, and Steller (1963). They studied the effects of intracranial injections of the protein synthesis inhibiting antibiotic puromycin administered prior to training of white mice in an electrified 'Y' maze, and they reported disruption of long term retention. This research established a method of assessing the functional importance of protein synthesis in the acquisition and retention of learning by observing the effects of inhibition of such protein synthesis by inhibitory drugs.

This first report of the effects of a PSI on learning by Flexner et al. (1963) initiated a plethora of research that has resulted in a capacious literature. The PSI'S most commonly employed in such research have been the antibiotics anisomycin, puromycin, cycloheximide, and acetoxycycloheximide. These antibiotics each block protein synthesis at the translational stage but represent three distinct classes of drug. Anisomycin disrupts protein synthesis by affecting the translocation of the peptide chain on the ribosome. Its mode of action is to cause a defect in the joining of the 60S ribosomal subunit to a

smaller subunit to form the 80S ribosome believed to be necessary for translation to commence (van Venrooji, Vaneenben and Janssen, 1977). Puromycin also interferes with translation, but it does so by acting as an analogue of aminoacyl-tRNA. Consequently, it is bound to the growing peptide chain instead of tRNA and is tightly linked to the carboxyl end of the peptide. This halts further translation, and the incomplete peptide with its attached puromycin is then released. These events would also dissociate the polyribosomes to monoribosomes. Cycloheximide and acetoxycycloheximide belong to the glutarimide group. They do not affect the formation of the complex between mRNA, tRNA, and the ribosome but interfere with the movement of the ribosome along the mRNA strand and thus are able to prevent the translation of the genetic code into the amino acid sequence. The modes of action of these drugs have been reviewed by Barraco and Stetter (1976) and Vasquez (1976).

A typical study of the effects of PSIs on learning involves an animal being trained during or shortly after drug injection, and retention of learning is then sampled at various post training intervals. Reviews of this area suggest that if cerebral protein synthesis is extensively inhibited during training, common basic findings emerge from research in rats, mice, goldfish, chicks, pigeons, quails, and in invertebrates such as praying manti, cockroaches, fruit flies, and marine slugs. These findings cover a range of learning paradigms as diverse as passive and active avoidance learning, shuttle box learning, appetitive and shock motivated learning, and long term habituation.

From this research, acquisition of the learning appears normal for initial training (Barondes and Cohen, 1966, 1967a, 1968a, 1968b; Barraco, Lovell & Eisenstein, 1981; Cohen & Barondes, 1968b; Flood, Bennett, Orme & Rosenzweig, 1975a; Gibbs & Ng, 1978; Gibbs, Roberts & Hambly, 1977; Squire & Barondes, 1974). However, performance of the task at retest days or weeks after initial training is worse in drug treated animals than in controls. This suggests impaired long term retention of the learning (Agranoff, 1968; Daniels, 1971, 1972; Davis, Rosenzweig, Groves, & Bennett, 1984; Davis, Spanis & Squire, 1976; Flood, Rosenzweig, Bennett & Orme, 1974; Flood, Bennett, Rosenzweig, & Orme, 1972; Flood, Bennett, Orme, & Rosenzweig, 1975a; Gibbs & Ng, 1977; Mark & Watts, 1971; Peterson & Squire, 1977; Rose, Gibbs & Hambley, 1980; Squire & Barondes, 1974; Squire & Davis, 1975). Animals in such experiments exhibit normal performance for a period of time after training with some variance between subjects, but retention becomes progressively impaired depending upon extent of training (e.g. Barondes & Cohen, 1968a, 1968b; Bromn & Noble, 1967, 1968; Cohen & Barondes, 1968a, 1968b; Davis, Rosenzweig, Bennett & Orme, 1978; Flood, Bennett, Rosenzweig & Orme, 1972; Gibbs & Ng, 1978; Jaffe, 1980; Pruzan, Applewhite & Bucci 1977; Quinton, 1974; Squire & Barondes, 1974; Watts & Mark, 1971a, 1971b).

However, there have been results which are at odds this summary of the main effects of PSI investigations. For example there are reports of memory recovering with time after the PSI effect (Barondes & Cohen, 1967b; Quartermain &

McEwen, 1970; Schmaltz & Delerm, 1974; Serota, 1971; Squire & Barondes, 1972b). Furthermore, some researchers have reported that the amnesic effect of these drugs may be increased by further treatments just after training or just before retest (Barondes & Cohen, 1968a; Davis et al., 1978; Flood, Jarvick, Bennett & Orme, 1976; Flood, Jarvick, Bennett, Orme & Rosenzweig, 1977a; Flood, Vidal, Bennett, Orme, Vsquez, & Jarvik, 1978; Quartermain, 1976; Quartermain & Botwinick, 1975; Quartermain, McEwen & Azmitia, 1970, 1972; Serota, Roberts & Flexner, 1972; Squire, 1979). Similarly, it has also been suggested that protein synthesis inhibition some time after training may cause amnesia but in general these effects appear to be not as pronounced as when inhibition is initiated immediately prior to or during training (Barondes & Cohen, 1968b; Davis, Rosenzweig, Kinkade & Bennett, 1981; Geller, Robustelli, Barondes, Cohen & Jarvick, 1969; Gibbs & Ng, 1977; Neal, Klinger & Agranoff, 1973). A review of these papers also reveals an underlying relationship between training-treatment interval and the amnesic effect in that, as this interval increases, performance at retest becomes increasingly more resistant to the amnesic effect.

Further to the above point, evidence has also been cited which suggests that further pharmacological manipulation after treatment with PSIs can retrieve PSI impeded learning. Such results have only been reported for experiments involving puromycin and evidence is limited to rodents. Flexner and Flexner (1967) reported intracerebral injections of saline restored memory at a 60 day retest in



mice treated with puromycin days after training, but similarly treated mice fail to attenuate the amnesia induced by pre-training injections of either puromycin or acetoxycycloheximide (Flexner & Flexner, 1968a; Rosenbaum, Cohen, & Barondes, 1968). This reported recovery with saline from post-training treatment has led some researchers to question the value of puromycin in this learning research (e.g. Barraco & Frank, 1983). They suggest that amnesia in rodents following delayed injections of puromycin is not due to inhibition of protein synthesis but, instead, results from some, as yet unspecified, action of peptidyl-puromycin at nerve endings. These problematic results with puromycin necessitate further investigation of this prolifically utilised amnestic agent.

Research using PSI's has generated a prolific, if equivocal, literature which has been extensively reviewed (Agranoff, 1971, 1980, 1981; Barondes, 1975; Barraco & Stettner, 1976; Dunn, 1980; Flood & Jarvick, 1976; Gibb & Ng, 1977; Quartermain, 1976; Rainbow, 1979; Roberts & Flexner, 1969; Rosenzweig, 1984; Squires & Barondes, 1972a; Davis & Squire, 1984). The apparent internally consistent conclusions derived from such studies are only as strong as methodological weaknesses allow and it has been argued that such weaknesses undermine proposed gene expression models of learning and memory (Goelet et al., 1986; Montarolo, Castellucci, Goelet, & Kandel, 1986). Such theoretical interpretations of PSI data are reviewed later.

One major criticism levelled at this research centres around the possible side effects of these powerful PSIs.

Agranoff (1968) and Barondes & Squire (1972) report inhibition of cerebral protein synthesis at a level greater than 80% to 90% can be achieved without gross changes in general behaviour of the animal. However, like any other drug, PSI's have more than one action on a biological system. Accordingly, when considering the effects of PSI's on learning and memory we must enquire if the observed effect in the behavioural assay could be due to some drug action other than inhibition of protein synthesis which may be required for memory.

The drug side effects problem can be divided into two main issues. First, the observed amnesic effect may be due to depletion of brain proteins other than new proteins needed for memory. Second, the drugs may affect some aspect of physiology to produce behaviour in the animal that is conducive to a misinterpretation in terms of amnesia.

One way that protein synthesis inhibition may cause amnesia, other than by inhibiting synthesis of new proteins needed for memory, is by depleting levels of constitutive proteins or by a build up of some metabolite resulting from such depletion. This possibility appears unlikely. Proteins in the brain are not only rapidly synthesised but are also rapidly degraded. While this process differs for individual protein species, its rate appears rather uniform throughout brain tissues. Half-life estimation of brain proteins is made difficult by transportation of newly synthesised proteins around the brain areas by axoplasmic flow, and this contributes to the loss of locally produced proteins which can erroneously lower estimates of specific protein

half-lives. Despite this problem of measurement, some estimates of the half-lives of brain proteins can be made, and they do appear remarkably short with approximately 0.65% of all proteins being replaced each hour. The range of protein turnover rates appears to be from a few hours to 10 days (Lajtha, 1976).

Since inhibition of protein synthesis for only a few hours by anisomycin, cycloheximide or acetoxycycloheximide produced amnesia (Barondes & Cohen, 1968b; Flood et al., 1974; Squire & Davis, 1975), it is unlikely that such short periods of inhibition could substantially deplete constitutive proteins with a half-life in the order of days (Barondes & Dutton, 1972). Of relevance here is the evidence that injections of acetoxycycloheximide 18 hours prior to training did not produce amnesia, even though cerebral protein synthesis was inhibited by 90% to 95% for 5 to 6 hours and by 50% to 60% at time of training (Barondes & Cohen, 1967b). This treatment should cause a greater loss of proteins with half-lives in the order of days and more extensive accumulations of metabolites than the amnesic dose of Cycloheximide or Anisomycin applied 20 to 30 minutes prior to training, and which inhibits protein synthesis for a few hours.

The most common drug paradigm employed is to give the PSI between 20 and 30 minutes prior to training. It could be argued that this procedure could deplete a short half-life, constitutive protein. While this issue has not been extensively researched, comparisons of the relative amnesic effects of a large dose of PSI given two hours prior to

training, and a smaller dose given minutes before training revealed that the first condition did not produce the amnesic effect despite calculations which showed animals in this condition had a greater depletion of short half-life proteins. This result has been demonstrated for anisomycin (Davis, Rosenzweig, Bennett & Squire, 1980) and with Cycloheximide (Squire & Barondes, 1976). The above results suggest that the critical factor for producing amnesia is not the levels of constitutive proteins present during or after training, but that it is the degree of inhibition of protein synthesis established at the training which is important. This issue remains to be resolved.

Much of the evidence could be criticised for its lack of adequate behavioural controls. It could be argued that the learning paradigms employed were not fully established as learning. Furthermore, no comparative data has been provided for the effects of early PSI activity on initial training or the effects of PSI on non trained groups at retest. Thus, adequate controls for short or long term performance effects of the drugs have not been reported.

Some researchers have also reported behavioural and physiological side effects of PSI's which may influence the interpretation of results. Locomotor activity has been shown to be affected by anisomycin, cycloheximide, and puromycin. Flexner and Flexner (1975) reported puromycin produced periods of lethargy up to two days post training followed by periods of excitability. Segal, Squire, and Barondes (1971) and Squires et al. (1970) reported that cycloheximide increased locomotor activity for approximately 30 minutes

post injection and then depresses it. Similarly, Squires and Barondes (1974) reported that subcutaneous injections of anisomycin suppressed locomotion for 1 hour post injection. These effects can be indirectly differentiated from the amnesic effects of these drugs by evidence that analogues of the PSI anisomycin which share the drugs action on activity do not produce the amnesic effect (Squires and Barondes, 1974). Also, Gutwein et al. (1974) showed that paragyline, at a dose that mimics the effects of cycloheximide on locomotion, did not produce amnesia. Furthermore, isocycloheximide, a stereoisomer of cycloheximide, does not inhibit protein synthesis but does disrupt locomotion. This drug was found to have no amnesic effect (Segal et al., 1971). Similarly, these researchers also report that amphetamine given in doses that prevent the amnesic effect of Cycloheximide does not attenuate the effects of the drug on activity. From this work, an account of the PSI effect in terms of locomotor changes appears inadequate. However, a more direct investigation specifically aimed at locomotor effects of more than one PSI at more than one dose in the same species is required.

Other side effects of PSI's such as, decreased food and water intake, diarrhea, and piloerection have also been reported (Davis, Rosenzweig, Groves, & Bennett, 1980; Flexner et al., 1966; Squires & Barondes, 1974; Squire, Geller & Jarvik, 1970). The possibility remains that such effects post training or at retest could contribute to an apparent amnesic effect. Barondes and Cohen (1968b) reported that subcutaneous injections of acetoxycycloheximide,

cycloheximide, and anisomycin produce symptoms of sickness in rats several hours after training. The major control for sickness effects remains that of injecting animals at some time after training or just prior to test. This has been reported not to produce the amnesic effect while still producing side effects (Agranoff, Davis, & Brink, 1965; Berner, Kesner & Partlow, 1978; Daniels, 1971; Eichenbaum, Quenon, Heacock & Agranoff, 1976; Flood, Rosenzweig, Bennett & Orme, 1973; Mark & Watts, 1971; Mayor, 1969; Squire & Barondes, 1974; Squire & Davis, 1975; Stettner, Barraco, Klauenberg, & Normile, 1979).

Sickness effects at time of training could also account for the amnesic effect of the drugs particularly as most studies involve injection of PSI at short intervals prior to training. This possibility has been studied directly by manipulating the degree of protein synthesis inhibition in terms of extent and duration (Davis et al., 1980), and results suggest that amnesia was related to the extent of inhibition of protein synthesis at the time of training and not to degree of sickness present during training. Unfortunately, sickness was only rated by the experimenters. Therefore, direct measure of the behavioural effects of sickness is required (see Chapter 8).

General and specific physiological effects of PSI's have also been suggested as possible mediators of observed amnesic effects. Puromycin is known to produce abnormal cerebral electrical activity (Cohen & Barondes, 1967). It has been argued that such disturbances cannot account for the observed amnesia in studies using other PSIs because the

glutarimide derivatives have no such effect. However, more recent research shows that these PSI's do affect cerebral activity in mice (Wilcox, Andry, & Luttges, 1974). Other potential physiological effects suggested as alternative explanations for the amnesic effects observed have included suppression of adrenal steroidogenesis (Garren, Davis, Crocco & Ney, 1966; Garren, Ney & Davis, 1965), increase in brain tyrosine levels, and a decrease in tyrosine hydroxylase with a consequent decrease in catecholamine levels (Barraco & Stettner, 1976; Bloom, Quinton, & Carr, 1977; Day, Overstreet & Schiller, 1977; Flexner et al., 1963; Flexner & Goodman, 1975; Gold & Sternberg, 1978; Lundgren & Carr, 1978; Quartermain & Botwinick, 1975; Quartermain, Freedman, Botwinick, & Gutwein, 1977). This has become one of the major alternative explanations of antibiotic induced amnesia, and the debate is extensively reviewed by Davis and Squire (1984).

More esoteric side effects of PSIs have included mitochondrial swelling with a dilation of cisterns of rough endoplasmic reticulum, inhibition of DNA synthesis, decrease of proteolytic enzymes, and inhibition of cyclic adenosine monophosphate phosphodiesterase (independent of its effect upon protein synthesis). However, most of these effects have only been observed with puromycin, and so they do not necessarily apply to other PSI's.

The issues of side-effects and alternative hypotheses of amnesic effects necessitates further investigation. All the above effects would produce changes in the natural behaviours of the test animals. Consequently, the

development of behavioural test batteries to screen PSIs for side-effects may provide a possible resolution to these issues. The development of such a test battery is discussed in Chapter 8 with a further discussion of these criticisms of PSI work.

Further to the above issues, a common trend in this area is to employ elegant biochemical analysis on a piece of learning behaviour that is inadequately established. The measurement of performance on any given learning task can only demonstrate an effect on the particular measure employed. Consequently, effects of pharmacological agents on performance cannot be distinguished from side-effects or alternative interpretations of results. It is necessary, therefore, to establish that the chosen behaviour is learning and to explore its parametric characteristics thoroughly (see Chapters 3 to 6).

Interpretation of the literature on PSI effects on learning and memory is further impeded by the diversity of animals, injection sites, dose of drugs, and time of injection in relation to training and retest which have been employed. Consequently, any conclusions drawn are a result of comparisons of results between research groups using vastly different learning paradigms, drug regimes and often in dissimilar species. A more comprehensive enquiry into the behavioural effects of PSIs on learning and general behaviour in one animal species with one learning paradigm is required, and it is to this end that this thesis is addressed.



In recent years the interest in possible interactions with the genome in learning has drawn heavily on the PSI generated literature. Recent evidence has concentrated on one of the simplest forms of learning; namely, habituation in the marine slug *Aplysia*.

The suitability of habituation as a model form of behavioural plasticity for investigation of the effects of protein synthesis inhibition is reviewed in Chapter 2. The extensive, mechanistic account of short and long term habituation provided by Kandel and associates has included investigations of the effects of PSIs on habituation in isolated and re-aggregated nerve cells and ganglia. Schwartz, Castellucci, and Kandel (1971) suggested that inhibition of protein synthesis by the antibiotics anisomycin, sparsomycin, or pactomycin during training had no effect on short term habituation in the isolated abdominal ganglion. In contrast, Squires and Becker (1975) reported that anisomycin did prevent long term habituation of the lick suppression response to distress calls in mice. Peterson and Squire (1977) performed a similar experiment but controlled for the possibility of drug induced conditioned gustatory aversion. Again, inhibition of protein synthesis prevented long term habituation. Unfortunately, neither Squires and Becker nor Peterson and Squire reported any short term data. Similarly, Schwartz et al., (1971) failed to report any long term data. However, taking these three studies together, they do suggest that protein

synthesis may be important in the production of long term habituation but not short term. Thin though this evidence is, habituation has enjoyed a prominent role in recent theories of learning mechanisms, and, in particular, in the suggestion that gene expression may be implicated by PSI results on long term habituation.

### 1.3 GENE EXPRESSION AND LEARNING

Interest in PSIs has largely been rekindled by the work of Kandel and his associates on the involvement of activity dependent gene expression as a mechanism of long term habituation and sensitization. Here, direct empirical evidence has been derived from PSI research. Rayport and Schacter (1986) and Montarolo, Castellucci, Goelet, Kandel, and Schacher (1985; 1986) reconstituted the monosynaptic sensory neuron to the motor neuron pathway of the *Aplysia* withdrawal reflex in a dissociated cell culture. This pathway is known to show both short and long term sensitisation (Frost, Castellucci, Hawkins, and Kandel, 1985). They have shown that the single synapse in the pathway undergoes a short term enhancement of EPSP amplitude in response to a single 5 min exposure to serotonin. These authors have also shown that the long term enhancement of sensitization induced by serotonin is blocked by exposure of the preparation to PSIs (anisomycin or emetine) or RNA synthesis inhibitors (actinomycin or alpha-amanitin) while short term enhancement is unaffected by such treatments (Montarolo et al., 1986).

The employment of PSIs on short and long term habituation in re-aggregated cells of *Aplysia* nervous system has been cited as the main empirical support for a gene expression model of long term learning (Kandel & Schwartz, 1982; Goelet, Castellucci, Schacter & Kandel 1986; Kandel et al., 1987). Although they were not the first to put forward such a theory, Kandel's research group have suggested a mechanism of short term habituation that does not require the synthesis of new proteins. Further, they have extrapolated from this mechanism to suggest gene expression as a component of long term habituation and supported such a model with PSI evidence from the dissociated cell preparation. They suggest that short term learning requires second messenger-mediated covalent modifications of previously synthesised proteins that modulate the properties of nerve cells and their synaptic connections (Kandel, 1978; Schwartz, Castellucci & Kandel, 1971). Inhibitors of protein synthesis were reported to have no disruptive effect on an in-vitro model of short term habituation. This result led Kandel and his co-workers to suggest that short term habituation in *Aplysia* involves the activation of receptor-linked enzymes by neurotransmitters which were responsible for the synthesis of intracellular messages. These second messengers, such as Cyclic adenosine monophosphate (cAMP), activate protein kinases that phosphorylate constitutive proteins required for plasticity. Retention of such changes would depend on the duration of the covalent modification of the constitutive protein and the maintained activity of the enzymes responsible for

second messenger synthesis.

Originally, Kandel and associates did not report data on the effects of PSIs on long term habituation in *Aplysia*. Recently this omission has been rectified with the report that transient blockage of protein synthesis in *Aplysia* during a 2-hour training period was sufficient to block retention of long term habituation tested 1 day later. Furthermore, inhibition of both protein and mRNA synthesis after training had no such effect (Montarolo, Castellucci, Kandel & Schacher, 1985; Schacher, Montarolo, Castellucci, Kandel & Goelet, 1986). This research was conducted in-vitro using identified re-aggregated *Aplysia* neurons as outlined above. Kandel draws on these results, along with results from the several hundred papers that have used PSI's in intact animals, to suggest a triarchical process view of memory with gene expression being suggested as a mechanism for long term memory lasting days in *Aplysia*. Further, the theory goes on to hypothesize a mechanism for very long term retention of learning (over 1 day in *Aplysia*) in which early regulator genes lead to a maintained alteration in the expression of late, effector genes. While Kandel's mechanism for short term habituation would not require changes in protein synthesis, both forms of long term habituation (retention of 1 day or more, and retention in terms of weeks) would require protein synthesis. Kandel's theory and its implications are further discussed in Chapter 19.

This elegant theory could, however, be criticised in that the invertebrate in-vitro model of learning does not represent true learning (e.g. Alkon, 1981). Similarly, any

gene expression model of learning is dependent upon a conclusive demonstration of protein synthesis inhibition effects on such learning. As outlined above, any review of the relevant literature reveals that the technique of PSI remains problematic (Thompson, 1987).

In support of Kandel, there are many potential biochemical mechanisms by which gene expression could conceivably be altered as a result of learning, and, more specifically, in a way conducive to a gene expression involvement in behavioural plasticity. For example, a qualitative change in gene expression (i.e., an expression of a novel protein) might arise from transcription from a previously untranscribed gene, a change in the processing (splicing) of a previously transcribed primary mRNA, or a change in efficiency with which an existing mRNA is translated into protein. Although examples of the regulation of eukaryotic proteins are known by all the above mechanisms, the one which has received most attention is regulation at the level of gene transcription. Accordingly, many mechanisms have been suggested to account for how neural activity might affect gene expression. Pertinent models of activity-dependent regulation of gene expression have included changes in Acetylcholine receptor (Klarsfeld & Changeux, 1985), Muscle myosin (Barton & Buckingham, 1985; Buller, Eccles, & Eccles, 1960; Henning & Lomo, 1985; Pette, Muller, Leisner, & Vrbova, 1976; Pette & Vrbova, 1985), alterations in catecholamine-synthesising enzymes (Faucon Biguet, Buda, Lamouroux, Samolyk, & Mallet, 1986), and also from growth factor changes (Covault & Sanes, 1985; Sanes &

Lawrence, 1983; Sanes, Schachner, & Covault, 1986).

In light of the recent theoretical impetus on gene expression, activity dependent changes in gene expression and the consequences of such processes for a mechanistic account of long term learning, a demonstration that protein synthesis is involved in learning becomes of critical importance to this area. Such inquiry requires evidence from a variety of sources and techniques briefly mentioned. Perhaps above all other, overcoming the methodological and perceptual weaknesses of PSI research and demonstrating their effects on a well established piece of learning in an intact animal is of critical importance to demonstrate new protein synthesis as a result of learning.

#### 1.4 AIMS OF THESIS

The initial programme of research aimed to establish a suitable form of learning and a methodology for its study. The learning behaviour chosen was short and long term habituation of the dorsal antennae withdrawal response of the terrestrial snail *Helix aspersa*. This programme of study established the behaviour as true learning and investigated the parametric characteristics of the phenomenon. Further experimentation in this phase provided behavioural evidence concerning differential process theories of habituation and are thus of consequence to PSI research (See Chapters 3 to 6).

A second programme of work goes on to investigate the effects of protein synthesis inhibition on short and long

term habituation in the intact snail. This programme consisted of several phases to address the multifarious issues of the area. First, a battery of behavioural tests was developed to study the effects of several antibiotics on general snail behaviour in order to investigate drug side effects. Second, a methodology of drug administration that did not affect learning was developed. Third, dose response effects were investigated as related to the main amnesic effects of the drugs. Last, three antibiotics at suitable doses screened by the test battery were compared for effects on short and long term habituation (see Chapters 7 to 12).

A third programme of experiments investigated the PSI injection time/behavioural effect relationship, to determine when, and for how long PSIs could disrupt long term habituation (see Chapter 13).

The final programme of research investigated the parameters of observed PSI effects in long term habituation and investigates any effects the drugs may have had on short term habituation. This was done by investigating any effects of the PSI's on the parametric characteristics of short term habituation in the snail which had been established in the initial programme of research (see Chapters 14 to 16).

Results of the four programmes of research are then discussed in light of Kandel's theoretical mechanisms of short and long term habituation and a three process theory of habituation in the snail is suggested (see Chapters 17 and 18).

The following chapter reviews the suitability of habituation as a form of learning with which to investigate

the effects of PSIs on short and long term learning.



## CHAPTER 2

### HABITUATION

Before any investigation of the effects of protein synthesis inhibitors on learning can commence, a suitable form of learning must be established. In light of the contentious nature of the PSI literature, a suitable learning behaviour must show both short and long term components, have well established and observable parametric characteristics, be unequivocally established as a form of learning, and be easily reproduced both within and between laboratories.

One of the simplest forms of learning, and one which meets the above criteria, is habituation. That is, the adaptive process by which animals learn to selectively ignore innocuous or irrelevant stimuli. It is defined as a decrement in responses to an initially novel stimulus with repeated presentations of that stimulus (Thompson & Spencer, 1966). Further, the mechanisms of habituation have been well researched at both the cellular (e.g. Kandel, 1987; Kandel & Schwartz, 1984; Schwartz & Kandel, 1982) and theoretical (e.g. Oman, 1979, Wagner, 1978, 1979, 1980, 1981; Whitlow & Wagner, 1984) levels.

Habituation is a ubiquitous phenomenon displayed by all reflexes apart from a few monosynaptic cases. The parametric characteristics of this form of learning were clearly defined by Thompson and Spencer (1966) and restated by Groves and Thompson (1970). These authors also list

parametric characteristics of habituation, and these must be established to demonstrate that a decrement of a response with repeated stimuli is true learning rather than sensory adaptation, motor fatigue, or a refractory process. There should be a decrement in response over iterated presentations of a stimulus. The response should recover with the presentation of a changed stimulus. Dishabituation should also habituate with repeated presentations of the dishabituating stimulus (habituation of dishabituation). Habituation of the response should show long term retention. It should also show stimulus frequency effects in that shorter interstimulus intervals should produce faster habituation in the short term. These characteristics show a remarkable commonality across phyla (Harris, 1943).

It has been argued that the supposed transient nature of habituation differentiates it from true learning (Evans, 1973; Hilgard & Marquis, 1940; Rosvold, 1959; Van Egeren, 1971). However, the classification of habituation as purely a short term phenomenon is not in accord with the literature. Indeed, for Thorpe (1956), the relative permanence of habituation was a definitive property. As stated above, Thompson and Spencer (1966) and Groves and Thompson (1970) also include long term effects as one of the parametric characteristics of habituation. They concluded that in a series of habituation training and response recovery, habituation becomes successively more rapid. This potentiation of habituation presupposes a relatively long term effect of training. Perhaps the existence of long term habituation above all else establishes habituation as true

learning.

2.1

EVIDENCE FOR LONG TERM  
RETENTION OF HABITUATION

Long term effects of habituation training have been reported in a multitude of studies which cover a wide range of species, responses, and recovery intervals. At one extreme, the response to light of the liver fluke recovered in a matter of seconds (Miller & Mohoffy, 1930). In contrast, Griffith (1924) reported incomplete recovery of the nystagmus response to rotation in humans who were tested 4 years after training. In between these extremes, there have been a large number of studies which have reported long term effects.

Prosser and Hunter (1936) concluded that habituation of the startle response and spinal reflexes in the white rat was semi-permanent in that it persisted for many minutes. Similarly, the strike response of the praying mantis recovered after only 1 hour (Rilling, Mittelstaedt, & Roeder, 1959). Retention in terms of hours has also been reported in the prey orientation response of the toad (Ewart, 1967), and the head shake response of the rat (Leibrech and Askew, 1969). The latter reported retention for at least six hours post training, and Clark (1960) reported retention seventeen hours post training in the withdrawal response of the marine ragworm Nereis pelagica.

Even longer retention (over 24 hours) has been reported in the courtship display of the insect Mormoniella

vitripennis (Barras, 1941), the death feigning response of the beetle *Tychius picrostris* (Bleich, 1928; Du Porte, 1916) and the withdrawal response of molluscs (Nagel, 1894; Parker, 1917; Pieron, 1909). Such retention intervals have also been reported from vertebrates in the autonomic responses of dogs (Dykman, Mack, & Ackerman, 1965; Konorski, 1948; Soltysik, Jaworska, Kowalska & Radom, 1961), cats (Wickens, Niels, & Wickens, 1966), rats (Glaser & Griffin, 1962) and humans (Bishop & Kimmel, 1969; Corah & Stern, 1963; Duffy & Lacey, 1946; Farmer & Chambers, 1925; Greenwood & Lewis, 1959; Griffin, 1963), and the alpha blocking response in humans (Popov, 1953).

Evidence from vertebrate studies shows retention lasting days from research on the aggressive display of *Betta splendens* (Clayton & Hinde, 1968), the response to shadow in guppies (Russell, 1976), the optomotor response of turtles (Hayes, Hertzler & Hogberg, 1968), the mobbing response of chaffinches (Hinde, 1960), the response to song of the white-crowned sparrow (Petrinovich & Patterson, 1979), the acoustic startle response in rats (Moyer, 1963), and autonomic responses in humans (Davis, 1934; Galbrecht, Dykman, Reese, & Suzuki, 1965). Evidence of such retention in invertebrates is limited to the contraction response of the earthworm (Gardner, 1968) and flat worms (Van Deventer, 1967), the escape reflex of the worm *Braschiomma* (Krasne, 1965), the sucker release response of the leech *Macrobdella decora* (Ratner, 1972), and the gill withdrawal reflex of *Aplysia* (Peretz & Moller, 1972). Further, habituation has been retained for 1 week in *Aplysia* (Castellucci et al.,

1973).

Habituation has been demonstrated to be retained for at least one week in the predation response of goldfish (Peeke & Peeke, 1972a), the head withdrawal response of the turtle (Hayes & Saiff, 1967), rotational nystagmus in cats and dogs (Collins & Updegraff, 1966), the cross scratch reflex in cats (Kozak, Macfarlane and Westerman, 1962), the approach response in rats (Denny & Leckart, 1965), and the autonomic responses in humans (Harding & Rundle, 1969; Kimmel & Goldstein, 1967; Porter, 1938; Frankenhaeuser, Froberg, Hagdahl, Rissler, Bjorkvall, & Wolff, 1967; Roessler, Collins & Burch, 1969; Stern, Surphlis, & Koff, 1965).

Retention over longer periods has also been reported. Retention 2 weeks post training was evidenced in the aggressive display of the paradise fish (Brown & Noakes, 1974), the response to song of the white crowned sparrow (Patterson & Petrenovich, 1979), and the skin conductance response in humans (Montagu, 1963), over 3 weeks for the aggressive display of the cichlid fish (Peeke, Herz, & Gallagher, 1971) and rotational nystagmus in rabbits (Lumkin, 1927) and humans (Guedry, 1965), 4 weeks in the electroencephalographic arousal response in rats (Leaton & Buck, 1971) and the tonic immobility response of chickens (Nash, Ronci and Girdaukas, 1976), 7 weeks in the contraction response of the flatworm *Dugesia decotocephala* (Westerman, 1963), and 2 months in the galvanic skin response of humans (Rachman, 1960). Perhaps the most impressive evidence of the long lasting effects of habituation training has been provided by Leaton (1976) who

reported the retention of habituation of the startle lick suppression response of albino rats for 30 days. This was achieved with only one 2-sec exposure to the stimulus during training.

The above research gives a clear indication that, from a variety of species, there is overwhelming evidence of long term habituation. In light of such long term effects, it seems highly unlikely that the decrements described could be attributed to other phenomena such as sensory adaptation and motor fatigue, and they provide strong evidence that habituation is true learning.

## 2.2 LONG TERM HABITUATION IN INVERTEBRATES

Evidence of very long term habituation from invertebrates is conspicuous by its absence, with only two reports of retention in excess of one week (Westerman, 1963; Carew and Kandel, 1973) (see earlier review). Harris (1943) and Humphrey (1933) have intimated, that in simpler organisms and simple responses in higher organisms, long term habituation occurs only after a very large number of stimulus presentations.

Unfortunately, interpretation of invertebrate literature purporting to demonstrate long term habituation is difficult, since much of it is typified by poor behavioural controls and weak statistical analysis.

It can, however, be concluded that habituation occurs in invertebrates and when considered with the vertebrate literature it appears to have both short (acquisition, a

decrement in responses) and long term (retention over time) forms, and it is sufficiently well defined to be a suitable learned behaviour for an investigation of the effects of protein synthesis on learning.

### 2.3 THE DIFFERENTIATION OF SHORT AND LONG TERM EFFECTS

There is some disagreement as to whether short and long term habituation have different underlying processes or merely reflect temporal differences in the same process. Clearly, if there is a real distinction between short and long term habituation, any evaluation of the effects of protein synthesis inhibition on habituation would be of great value and must take such differences into account. The PSI research on habituation reviewed in Chapter 1 (Peterson and Squire, 1977; Schwartz et al., 1971; Squires & Becker, 1975) is pertinent to this issue. This research suggests that short term habituation is protein synthesis independent while long term habituation requires the synthesis of proteins. This has culminated in the conclusions that different processes mediate short and long term habituation (Goellet et al., 1986; Kandel & Schwartz, 1982; Montarolo et al., 1986)

Earlier, Galbrecht et al. (1965) concluded that short and long term habituation were mediated by the same process. However, this conclusion was based on the rather weak evidence that the shape of the inter session function was similar to that of the intra session function. Thompson and Spencer (1966) reached the same conclusion. Although they

admitted the possibility that two processes may be necessary to account for short and long term habituation, they suggested that the similarity of the parametric features of the two forms of habituation implied some identity of underlying process. While Horn (1970) has attested to the behavioural dichotomy of short and long term habituation, he has suggested that this distinction may not reflect discontinuous processes in the nervous system.

Many authors have gone further than Horn, and suggested, implied, or required a clear distinction between short and long term habituation (e.g. Askew, 1970; Buchwald & Humphrey, 1973; Castellucci & Kandel, 1976; Davis, 1970a; Goelet et al., 1986; Graham, 1973; Groves & Lynch, 1972; Hinde, 1954; Kohler, 1976; Leaton, 1976; Ohman, 1979; Peterson & Squire, 1977; Schwartz & Kandel, 1982; Sharples & Jasper, 1956; Sokolov, 1955, 1969; Wagner, 1976, 1978, 1979, 1981; Whitlow & Wagner, 1984).

On the basis of a series of studies of the mobbing response in chaffinches, Hinde (1954) made one of the earliest attempts to specify a systematic difference between short and long term habituation. The short term effects were characterised by relatively rapid response recovery, generalization to any stimulus which could evoke the response, a dependence on stimulus duration such that long stimuli produced greater habituation, a dependence upon stimulus intensity such that stimuli produce slower habituation, and the greater efficacy of massed presentations over those that are spaced. On the other hand, long term effects were thought to exhibit relatively slow



response recovery, less stimulus generalization, and a relative independence from stimulus duration and intensity. Further, spaced practice (SP) was claimed to produce more long term effect than massed practice (MP).

In a later publication, Hinde (1960) appeared to eliminate one of these differentiating factors. In this study, he reported a direct relationship between stimulus duration and response decrement for both short and long term habituation, using stimulus durations of 12, 24, and 48 minutes and 24 hours. Conversely, Thompson and Welker (1963) provided data which supported the differentiation of short and long term habituation in terms of stimulus duration effects. However, the results pointed to a different relationship from that originally posited by Hinde (1954). Thompson and Welker investigated habituation of the head orientation response in cats presented with auditory stimuli of 2 sec or 0.1 sec duration in five sessions spread over 1, 3, 10, or 28 days. They reported no within session difference between duration conditions, but in the 2 sec condition, there was no evidence of any long term retention of habituation, whereas, in the 0.1 sec condition, there was evidence of significant long term retention. Askew, Leibrecht, and Ratner (1969) provide one other attempt to investigate the effects of stimulus duration on short and long term habituation utilising the head-shake response in the rat with stimulus durations of 5 or 20 sec. Unfortunately, neither duration condition produced any significant retention of habituation over the long term interval employed (24 hours).

### 2.3.1 STIMULUS INTENSITY EFFECTS AND SHORT AND LONG TERM HABITUATION

Three studies have investigated the effects of stimulus intensity on short and long term habituation. Kuenzer (1958) studied habituation of the twitch response in earthworms. He used electrical stimuli of 150mV or 250mV and reported that worms in the 150mV condition required fewer trials to reach the criterion of habituation. However, in a long term test 24 hours later, the 250mV condition produced greater retention of habituation in terms of savings in trials to rehabilitate. Indeed, the 150mV group displayed virtually no retention.

In an investigation of habituation of the skin conductance response in humans, Ray (1979) also analysed trials to habituation and rehabilitation, but he did not express them as savings scores as did Kuenzer. In the short term test, subjects in a 50dB condition required fewer trials to habituation than did those in a 90dB condition, but in terms of trials to rehabilitation, there was no difference between groups in a long term test 1 week after the initial session. Further, as the 90dB condition produced significantly larger initial responses in this long term test, this equivalence of speed of habituation was achieved despite within session intensity effects. However, as subjects in the 90dB condition took longer to habituate in the first session, they necessarily had greater exposure to the stimulus than did those in the 50dB condition.

Similarly, in the study reported by Kuenzer (1958), the 150mV group received fewer stimuli than the 250mV group. Ray has argued that it was necessary to equate groups in terms of levels of habituation at the end of the first session, indeed, the habituation process is thought to proceed beyond the point of zero response (Smith & Council, 1978; Smith Dickel & Deutsh, 1978; Stephenson & Siddle, 1976; Thompson & Spencer, 1966; Waters & McDonald, 1976). Therefore, if subjects had been given a fixed number of trials in the first session, those in the 50dB condition could have reached a more profound level of habituation than those in the 90dB condition. In this situation, the requirement to equate groups in terms of level of habituation is necessarily in conflict with the requirement to equate them in terms of experience of the stimulus. Thus, the possibility cannot be ruled out that the long term effects reported by Ray (1979) and Kuenzer (1958) were due to differential exposure to the stimulus.

Askew (1970) equated subjects in terms of number of stimuli experienced. In this study, subjects were exposed to 10 trials at one of five intensities of a stream of air directed at the ear. After a 30 minute retention interval, they received a further five trials at the same intensity. Askew reported the short term effects in terms of rate of habituation, and he suggested that in the case of these data, this was effectively equivalent to a trials to asymptote measure. Such a measure would appear to be similar to the trials to habituation measure used by Kuenzer (1958) and Ray (1979), and it yielded similar results in that more

trials were required to reach asymptote by subjects in the higher intensity conditions. Unfortunately, Askew did not report long term effects in terms of this measure, but he did conclude that there was no effect of intensity on the initial recovery of the response.

One study has looked at the effects of stimulus intensity on generalization of habituation. Davis and Wagner (1968) reported more long term generalization with higher intensities during training. However, they did not report their short term results, and no other study has provided any comparable short term data.

Although the evidence is not as strong as it might first appear, the above experiments do suggest that short and long term habituation can be differentiated in terms of the effects of stimulus intensity. While lower intensities appear to produce more rapid habituation in the short term, high intensities appear to produce equal or greater long term effects.

Differences between short and long term habituation in the effect of stimulus frequency i.e. the massing and spacing of training, have been the subject of more extensive investigation.

### 2.3.2 THE EFFECTS OF MASSED AND SPACED TRAINING ON SHORT AND LONG TERM HABITUATION

Evidence of the effects of massed and spaced training on short and long term habituation can be divided into two sections. First, research that has manipulated Interstimulus

interval (ISI), and second other forms of massing and spacing of training. By far the largest body of evidence pertaining to differential effects of massing and spacing of training has arisen from manipulation of ISI. Experimental details are included in this section as an illustration of typical habituation paradigms and for further reference in the empirical chapters.

### 2.3.3 INTERSTIMULUS INTERVAL EFFECTS

Kinastowski (1963) studied habituation of the contractile response to mechanical stimulation in *Spirostmum* and demonstrated habituation with ISIs of less than 12 sec, but found no habituation with ISIs of 1 min. Wood (1970) investigated withdrawal to mechanical stimuli in *Stentor coeruleus*, gave each animal 60 stimuli at ISIs of 1, 2, and 3 mins. Animals in the 1 min condition displayed significantly greater amounts of habituation than those in the other conditions which did not vary significantly between each other. Jennings (1905) and Pieron (1908) found evidence of habituation in the phylum Coelenterata. Animals habituated with ISIs of up to 3 min but not with ISIs of over 5 min. Pieron (1910) also found a direct relationship between ISI and trials to habituation of the contractile response to shadow stimuli in *Littorina stagnalis*. Similarly, Ratner and Stein (1965) demonstrated an ISI effect on habituation of the withdrawal response of *Lumbricus terrestris*, reporting fewer responses over sixty trials with an ISI of 6 sec than with an ISI of 88 sec.

Greater disparity of ISI was used by Clark (1960) in a study of habituation in the Polychaete, *Nereis*. Worms in five groups were presented with sudden increases in light at ISIs of 30 sec, 1, 2, 3, 4 or 5 min. Clark reported a direct relationship between ISI and the number of trials required to reach complete habituation. Pieron (1913), investigating habituation in *Littorina obtusata*, demonstrated that the direct relationship between trials to habituation and ISI did not hold true for long term habituation.

Moving up to vertebrates, Prosser and Hunter (1936) demonstrated a similar relationship between ISI and habituation of the startle response in the white rat. Reporting that stimuli presented at a rate of one every 2 or 5 sec produced extinction (habituation) of the response in a much shorter time than stimuli presented at 10 or 15 sec intervals. However, it would appear that in this study ISIs may have been confounded with the number of stimuli presented (Davis, 1970a).

The effects of ISI on habituation have also been investigated in isolated biological preparations. Hom and Rowell (1968) used a visual interneuron of the locus *Tribocerebrum*. They presented 10 stimuli in each of three ISI conditions 120, 40, or 5 sec and found that the shorter the ISI, the greater the amount of habituation. Thompson and Spencer (1966) investigated the decline in responding in the saphenous nerve in the spinal cat. They concluded that there was a greater amount of habituation with an ISI of 1 sec than with ISIs of 3 or 2 sec. However in this study, the duration of session was held constant across condition,

possibly confounding ISI with the number of stimuli presented. Groves, Lee, and Thompson (1969), using the hind limb flexion reflex of the acute spinal cat, reported the same relationship for habituation when 200 stimuli were presented in one of five ISI conditions: 2, 1, 0.5, 0.25 and 0.13 sec. Farel and Thompson (1972) investigated habituation of the ventral root response to dorsal root stimulation in the isolated frog spinal chord using three ISI conditions 2, 5 and 10 sec. In each condition, there were 20 stimulus presentations. They concluded that the shorter the ISI, the greater the amount of habituation. Buchwald, Halas and Schramm (1965) investigated the effects of ISI on motor unit responses in the spinal cat. Although their manipulation of ISI involved four different intervals, the intensity of the stimulus was kept constant in only the 15 and 45 sec ISI conditions. The latter produced slower habituation than the former.

All of the above studies suggest that habituation is directly proportional to stimulus frequency. Two studies have produced results in conflict with the above. Griffin (1970) used six groups of decerebrate cats which received 500 stimuli at ISIs of 1, 5, 10, 20, 100, or 300 seconds. He reported that rate of habituation was directly related to length of ISI. Griffin and Pearson (1967) demonstrated a similar relationship between ISI and the number of stimuli necessary for the complete loss of the flexor withdrawal response in a conscious rat. Although these two studies have generated results contrary to the conclusions of all the above studies, there appears no dissonance of procedure

employed which could possibly account for such discrepancies. Nevertheless, the animal literature does display a remarkable level of agreement over a wide range of animals with short ISIs consistently reported as being more effective in the elicitation of habituation than long ISIs.

Coombs (1938) investigated habituation of the skin conductance response (SCR) to auditory stimulation, using a somewhat unusual design. Subjects received five presentations of a 100Hz tone at ISI conditions of either 15 or 30 sec. All subjects received a further 10 stimuli with a 30 sec ISI. The 15 sec group demonstrated greater habituation over the first five stimuli than did the 30 sec group. Indeed, the 30 sec group took 10 stimuli to reach the same level of response as the 15 sec group after five stimuli.

Winokur, Stewart, Stern, and Pfeiffer (1962) used a within subject design to look at the effects of different ISIs on habituation of the SCR to electric and auditory stimulation. Subjects were presented with 20, 5000Hz tones at varying ISIs; seven ISIs of 60 sec and four of 90 sec. They were subsequently presented with 20, 0.5m/amp shocks with the same sequence of ISIs. After the first few trials, the magnitude of response depended upon the ISI which preceded it: the shorter the ISI, the smaller the response. Towards the end of the series, the response was smaller if the ISI immediately before the stimulus was smaller than the preceding ISI. The same results were reported for shock stimuli, but the pattern took longer to emerge. Schaub (1965) suggested that the use of a within subject design



eliminates the possibility that the effects of ISI in between subject designs is due to the necessary increase in duration of the session with longer ISIs. In an experiment using ISIs of 30, 60, and 120 sec balanced over 18 presentations of a white noise stimulus, smaller SCRs were produced when the stimulus was preceded by a 30 or 60 sec ISI than when it was preceded by a 120 sec ISI. He also reported a between subject experiment in which 20 stimuli were presented with ISIs of 30, 60, or 180 sec. The 30 and 60 sec groups displayed smaller responses than did the 180 sec group. Further, there were progressively fewer evoked responses with decreasing ISI.

The argument that ISI effects are not due to differences in session duration is supported by Gatchel and Lang (1974) who used ISIs of 20, 60, and 100 sec, and by Geer (1966b). Both these studies found shorter ISIs produced smaller responses, and there was no interaction between ISI length and session duration.

While the direct relationship of ISI with short term habituation appears well established, the evidence concerning the effects of ISI on long term habituation is equivocal.

Pieron (1913) argued that a direct relationship between ISI and size of response does not hold for long term habituation. Pieron used a paradigm in which 15 shadow stimuli were presented to *Littonia obstusata* in seven ISI conditions ranging from 3 to 120 sec. After a 30 sec rest period the stimuli were again presented with the ISI conditions of the first training series maintained. There

was not a simple direct relationship between ISI and trials to habituation, as the most effective ISI found was 60 sec. Goodman and Weinberger (1973) investigated gill-beat suppression and heart beat interruption responses in *Necturus*. The three ISIs used were 30, 120 and 400 sec, and stimuli were presented in two 15 trial sessions which were separated by 75 to 90 min. Speed of habituation and amount of habituation decreased with increasing ISI. There appeared to be no difference between the first and second sessions. However, although heartbeat interruption responses demonstrated slower habituation with increasing ISI in the first session, only the 120 seconds group gave any heartbeat interruption responses in the second session.

In an attempt to provide more systematic evidence of the differential effects of ISI on short and long term habituation, Davis (1970a) studied the startle response in the white rat. In a prehabituation session, the rat was presented with a 4000Hz, 120dB tone 300 times with ISIs of 2, 4, 8, and 16 sec used randomly and equally throughout the session. In the habituation test twenty four hours later, subjects were allocated to either a 16 second ISI group or a 2 second ISI group, and the same tone was presented 1000 times. A posthabituation session which was identical to the prehabituation session was also administered. For half the subjects in each ISI condition, this posthabituation session took place 1 minute after habituation training, and for the other half, the delay was 24 hours. Davis argued that if the same ISI was used during the long term test as was used during habituation training, the short term effects of ISI

on responding during this test would contaminate any long term ISI effects. Consequently, Davis used the same ISIs for all conditions during the posthabituation session, so as to obtain an estimate of the long term effects of different ISIs during training without confounding them with the short term effects of the conditions in the test situations. However, it has been argued (Stephenson & Siddle, 1983) that this attempt to circumvent the confound means Davis' study does not investigate the long term effects of different ISIs during training on the amount or rate of habituation, rather it looks at the long term effects of ISI on generalization of habituation. Specifically, this is the ability to generalise from one, constant ISI condition, to a condition in which ISI varied over over four durations.

The prehabituation session yielded results in accord with those reported by Winokur et al. (1962) and Schaub (1965) in that longer ISIs prior to stimulation were more likely to be followed by a response. In the habituation session, animals in the 16 sec condition produced more startle responses throughout training. This difference was as marked on the second presentation of the stimulus as it was on the 1000th. However, in the posthabituation test, the 16 sec group produced fewer startle responses throughout all test intervals than did the 2 sec group, and there was a greater difference between groups after 1 min than after 24 hours. In support of the conclusions reached by Davis, the 2 sec ISI group did demonstrate recovery of the response after a 2 sec ISI in the posthabituation test, whereas, the 16 sec group did not show any response recovery after a 16

sec ISI in the posthabituation test. The effects of a change from a variable to a constant ISI and back again to a variable ISI cannot, however, be disentangled from any possible direct effect on long term habituation and recovery. Davis appears to have demonstrated a differential effect of ISI on long term generalization.

Similarly, Davis (1970b) reported an experiment with a prehabituation session consisting of 9 presentations of each of three tone intensities (100, 110 and 120dB) given in pseudo-random order with an ISI of 1 min. After 24 hours, subjects were divided into three groups for the habituation session in which they were presented with a 120dB tone. The first group received 1000 presentations with an ISI of 16 sec, the second group had an ISI of 2 sec and the third also had an ISI of 2 sec but received 8000 presentations of the stimulus. The posthabituation session was identical to the prehabituation session, and took place 5 min after the habituation session. The 16 sec group produced more responses per stimulus in the habituation session than did either of the 2 sec conditions. But, in the posthabituation test, the 16 sec group produced significantly fewer responses to all stimuli than did the 2 sec group which received 8000 presentations which in turn produced fewer than the 2 sec group which received 1000 presentations. Consequently it can be concluded that long ISIs produced greater retention.

Gatchel (1975) looked at the human SCR and heart rate (HR) responses (deceleration over the first three post-stimulus beats) to 65dB tones, he used a similar

paradigm to Davis. Subjects were presented with 15 stimuli in one of two ISI conditions, 20 seconds or 100 seconds. All subjects were presented with a further 15 trials with an equal number of 20 seconds and 100 seconds ISIs given in a random sequence after a 15 minute rest period.

Unfortunately, Gatchels statistical analysis was debilitated by not reporting the main effect of ISI, and the reported significance of the interaction between ISI and trials is no longer apparent when conservative degrees of freedom are applied (Greenhouse and Geisser, 1959). Examinations of the graphical presentations of these data indicate that the 100 seconds ISI group produced larger SCRs in the short term session, but showed less recovery and subsequently smaller SCRs in the long term session than the 20 second ISI group. Similar problems apply to the HR data. Again, examination of the graphical presentation of the data indicates smaller responses in the short term for the short ISI group, however, there appears to be no difference between groups in the long term session. These results appear to be in accord with those of Davis (1970a,b), that while short ISIs produced more short term habituation than long ISIs, they produced less long term generalization of habituation.

Askew (1970) studied the direct effect of ISI on short and long term habituation. Studying the head shake response in the white rat, he investigated the effects of 1, 10 and 100 seconds ISIs. The rats received 10 stimulus presentations of a given ISI. Then after a 30 minute break, they received a further five stimuli at the same ISI. As would be predicted, the longer ISIs produced more responses

over the first 10 stimuli. This was also the case with the last five long term stimuli. But, as Davis (1970a) pointed out, this was probably due to contamination by the powerful short term effects of ISI. However, the first response in the long term session was not affected by this confound, and here the efficacy of a long ISI in training was apparent. There was a large recovery of response during the 30 minute break in the 1 and 10 seconds groups but not in the 100 seconds group. So, the longer ISI in training produced more long term habituation measured in terms of recovery of response.

The effects of long term habituation of the skin conductance response (SCR) in humans, was investigated by Ray (1979) using 40 and 120 seconds ISI conditions. Unlike the above studies, Ray did not present a fixed number of stimuli during training. Subjects were presented with a 50dB tone until they reached a criterion of no response. After one week, they were again exposed to the stimulus until they reached the habituation criterion. The same ISI conditions were maintained in this second session. Contrary to the results reported by Askew (1970), there appeared to be no difference between groups in terms of the response to the first stimulus of the long term test. However, although the short ISI group required significantly fewer trials to reach habituation criterion in the first session, there was no difference between groups in this measure in the long term session. In this case, long ISIs appeared to be as effective in the production of long term habituation as did short ISIs despite the short term, within session effects of ISI during

the second session. It should be considered, however, that subjects in the long ISI condition received more stimuli during training than did the short ISI group. This may militate against the above conclusion due to differential stimulus exposure effects.

The one study to report the same effects of ISI in both short and long term habituation was reported by Szlep (1964). He studied the response of the spider *Araneus* to mechanical stimulation of its web, and reported that an ISI of 5 minutes retarded habituation within and across sessions relative to an ISI of 15 seconds.

In conclusion, long ISIs during training appear to produce more long term generalization of habituation than do short ISI. Furthermore, although the evidence is somewhat less conclusive, there is some indication that long ISIs during training result in less recovery of the response in a long term test and as fast, if not faster habituation during long term test than short ISIs. The relationship of ISI with short and long term habituation and the potential differential mechanisms remains to be resolved. The issue of ISI effects on short and long term habituation is discussed in Chapter 5.

These specific ISI results, as a form of spaced practice, pertain to the more general issue of the differential effects of other forms of massed and spaced training on short and long term habituation. Despite the generalisation<sup>n</sup><sub>A</sub> of research on ISI effects, research on other forms of massed and spaced training have not been reported in the same preparations used to investigate these ISI effects.

Investigations of both ISI and other distributions of training will be investigated in the snail (see Chapters 5 & 6). As outlined in Chapter 1, the effects of PSI on the parametric characteristics of learning rather than on performance on a learning task may usefully serve to advance protein synthesis inhibition as a useful technique.

#### 2.3.4 EVIDENCE FROM OTHER FORMS OF MASSED AND SPACED TRAINING

Ohman (1979) has postulated that forms of massing and spacing of training other than the manipulation of ISI should produce results similar to those found by manipulating ISI.

The literature on massed and spaced training effects in habituation studies highlight a definitional problem, that of researchers failing to agree what comprises massed and what comprises spaced training where variegated distribution of training has been employed.

In reviewing animal studies there are only two studies which have compared different distributions within a session. The most straight-forward of these was reported by Dyal and Hetherington (1968) who investigated habituation of the light avoidance response of the polychaete *Hesperanoe adventor* under two conditions of distribution of training. Subjects received either 20 trials in one block (massed) or two trials in each of 10 blocks which were separated by 54 minutes (spaced). This procedure was repeated on each of the next two days. They found that the groups did not differ



significantly on the first day, and despite the lack of statistical analysis of the data for the development of within session habituation on the subsequent two days, there appeared to be no difference between groups. However, there was a difference in terms of the between session retention of habituation in that the spaced training group displayed significantly less recovery of the response than did the massed training group.

Reporting a similar long term effect to that of Dyal and Hetherington (1968), using a very different distribution of training was a study by Hinde (1954), looking at the mobbing response of Fringilla coelebs. Subjects in the spaced condition were presented with the stimulus for three minutes once a day for five days. In the massed training condition, they received one 20 minute presentation, and were tested 24 hours later. For both groups, the number of responses in the test session were expressed as a percentage of the responses in the first session, and this was used as a measure of long term retention of habituation. The massed training group showed approximately half the retention displayed by the spaced practice group.

Danziger and Mainland (1954), investigated habituation of the exploratory behaviour in the albino rat. They compared the short term effects of a single exposure of stimulus with training distributed over days. Three conditions were sampled. A single exposure of 40 minutes, 6 minutes of exploration every day for seven days, and 2 minutes of exposure a day for 20 days. The single exposure group produced the least exploratory behaviour, and there

was a decrease between the first and last 6 minutes of training. The second group produced more exploratory behaviour with less of a decrease between the first and last six minutes. The group with the most spaced training produced most exploratory behaviour, and did not show a decrement across trials. Peeke, Herz and Gallagher (1970) also compared the effects of a single exposure with that of several exposures spaced over days, but only reported the short term effects of this type of differential presentation. Peeke et al. concluded that a single exposure of 24 to 48 hours was more effective in the reduction of biting and chin display responses of Cichlasoma nigrofasciatum, than 20 minute exposure per day for 38 to 44 days. The conclusion may be confounded however, as there was differential stimulus exposure between the massed and spaced conditions. The massed training condition received approximately twice the amount of stimulus exposure than the spaced conditions.

A somewhat complex form of massing and spacing of training was reported by Patterson and Petrinovich (1979) who compared the results of two experiments on habituation of responses to song in the White-crowned sparrow (Patterson and Petrinovich, 1979; Petrinovich and Patterson, 1979). In the spaced training experiment, subjects received stimuli of 2 second duration in blocks of 10. One minute and five minutes inter-block intervals were alternated for a total of 160 stimuli. There was a further 60 minute interval, and the above procedure was repeated for another block of 160 stimuli. In the massed training experiment, subjects

received the same stimulus as above in one block of 10 stimuli followed by another block of stimuli with an inter-block interval of 1 minute. After a further 1 minute inter-block interval, 100 stimuli were presented. There followed a 5 minute inter-block interval after which another 100 stimuli were presented. A subsequent 60 minute interval was followed by a further 100 stimuli in a single block. Thus, the animals in both conditions received 320 stimuli. The ISI was 11 seconds for all blocks of stimuli in both experiments. Patterson and Petrinovich compared the decline in response to the first 160 stimuli of the massed training experiment with those to the first 160 of the spaced training experiment. They concluded that spaced training produced a greater decline in response. However, examination of the graphical presentation of their data suggests that this apparent difference was due to the low initial levels of response in the massed training condition, which curtailed the potential for conflicting results for a decline in response to repeated stimuli.

Conflicting results with regard to short term habituation were reported by Peeke and Peeke (1970). They exposed mature, male *Betta splendans* to a conspecific male stimulus under two distributions of training. One group received 15 minutes exposure per day for 20 days (spaced training), the other received 60 minutes per day for 5 days (massed training). The biting and aggressive display response declined faster in the spaced condition. However, in habituation of the aggressive display of the paradise fish *Macropodus opercularis*, there was no difference between

two, 20 minute exposures a day for 5 days, and one, 40 minute exposure a day for five days. But, both these conditions produced significantly more rapid habituation than continuous exposure for 5 days (Brown and Noakes, 1974).

A different form of massing and spacing of training was investigated by Carew et al (1972) in an examination of the decline of the gill withdrawal reflex of *Aplysia californica*. To compare the effects of massed and spaced training on short and long term habituation. The sea slugs were randomly assigned to one of three groups: spaced training, massed training, and a control group that received no training. Sea slugs receiving spaced training were given 10 stimuli per day over 4 days. Sea slugs receiving massed training were given no stimulation on days 1 to 3; on day 4 they were given 40 consecutive stimuli. Despite the fact that both groups had been given the same number of stimuli, the sea slugs receiving spaced training showed significantly greater retention one day and one week after training than did those in the massed group. Sea slugs in the massed training group showed significant retention of habituation one day after training when compared to their responses on the previous day. But on the one week retest the massed training group showed no significant retention while the spaced training group did show significant retention. Suggesting, although both massed and spaced training groups showed identical short term habituation at the end of the fortieth trial, massed training is not retained as long as spaced training. Fearing (1940, 1941) used the same type of

spacing of training in investigations of the habituation of the post rotational nystagmus response in pigeons. Subjects in the massed training experiment (Fearing, 1940) were rotated for 30 seconds 61 times in one session, and tested for retention 56 or 96 days later. Pigeons in the spaced training experiment (Fearing, 1941) were rotated for 30 seconds 10 times a day for 14 days, separate sub-groups were subsequently tested at eight intervals which ranged from 16 days to 225 days. Fearing (1941) concluded that differential spacing of training had no effect on the acquisition of habituation, but that it did affect retention. There was no evidence of retention in the massed training group after 96 days, whereas there was evidence of retention in the spaced training condition after 225 days. While appearing to be in accord with the findings of Carew et al (1972), the value of the support is questionable as the two groups received such vastly different stimulus exposures due to different amounts of training.

This methodologically diverse literature can be interpretively unified to some extent as follows, studies which have compared training on one day, and studies spaced over several days. Fearing (1940,1941), Hood and Pfaltz (1954), Carew et al (1972) have consistently reported no effect on short term habituation, but if long term habituation data were analysed, spaced training conditions exhibited less recovery of the response than did the comparable massed trained conditions. Similarly, results were reported where training was differentially distributed within a session (Dyal and Hetherington, 1968) or across

sessions separated by a different number of days (Brown, 1965). With the complex form of differential distribution of training reported by Patterson and Petrinovich (1979), the relatively massed condition was reported to have produced less within session decrement, but it has been argued that this apparent difference may have been spurious. Two experiments which have compared a single long exposure with several short exposures spread over days reported that for short term habituation, massed training produced fewer responses than spaced training (Danziger and Mainland, 1954; Peeke et al., 1970). In the one study that reported long term effects (Hinde, 1954), spaced training produced less response recovery than massed training. Studies which have compared training comprising a single, short exposure per day spread over many days with that consisting of a single, long exposure per day spread over a few days have generated conflicting results. Danziger and Mainland (1954) reported that massed training was more effective than spaced training in producing short term response decrement, whereas Peeke and Peeke (1970) reached the opposite conclusion. The situation is still further confused since Brown and Noakes (1974), with a similar form of differential distribution of training, found no difference between relatively massed and spaced conditions, but both were more effective than continuous exposure.

With regard to the differentiation of short and long term habituation effects, the evidence from animal studies of massed and spaced training effects would appear to support the dichotomy, over the above range of stimulus

spacings, <sup>Thus,</sup> alluding to the conclusion of no effect on short term habituation, with spaced training conditions showing less recovery in the long term.

One underlying criticism of this reviewed research is that many investigators use only one measure of habituation. This measure being differential in comparative terms to those employed by other researchers, generating a need for a more comprehensive investigation of the phenomenon using different measures of habituation all in the same response system (see Chapter 6).

The above results would all lend support to a differential process theory of short and long term habituation. In light of the results of the few PSI studies that have employed habituation, these findings are of great importance to the interpretation of protein synthesis inhibition experiments. Establishment of parametric characteristics as outlined and reviewed in the above three sections in the same learning preparation as used in a PSI investigation, would serve to advance our understanding of both the role of protein synthesis in short and long term habituation and advance our understanding of the differential processes underlying these two phenomena..

#### COMMENT

Habituation appears an ideal 'vehicle' for an investigation of the effects of protein synthesis inhibition on short and long term habituation. It is an ubiquitous phenomenon, easily replicable, has well defined parametric

characteristics, and displays short and long term effects. Furthermore, investigation of PSI effects on short and long term habituation may serve to resolve the debate between differential versus single process theories of habituation. Having established that habituation is a suitable and interesting form of learning to employ, short and long term habituation must be unequivocally demonstrated in a suitable animal that is suitable to the demands of PSI research. A suitable response and animal is explored in the next chapter; namely habituation of the dorsal antennae withdrawal response in the common land snail Helix aspersa.



## CHAPTER 3

### ESTABLISHMENT OF A SUITABLE LEARNING BEHAVIOUR

Behavioural investigation of protein synthesis inhibition is extremely subject intensive. Consequently, experimental animals must be both economical and readily available. While habituation appears an ideal behaviour for such studies, a response that habituates in a suitable organism needs to be investigated, and a decrement in this response across iterated stimuli must be established as habituation. Preliminary studies revealed the common snail Helix aspersa as an eminently suitable candidate and the antennae withdrawal response a suitable, modifiable behaviour in the snail. This chapter reports experiments which establish a suitable methodology, demonstrate a decrement in response across repeated presentations of a stimulus, and demonstrate a change stimulus effect in order to establish this decrement as habituation.

Habituation of the antennae withdrawal response in this terrestrial snail is a particularly good exemplar of the supposed adaptive function of this form of learning. That is, an animal learns not to respond to repeated stimuli which are of little biological consequence (Peeke, 1984; Shalter, 1984). The snail is not well adapted for flight or fight, so when faced with potential danger, its main form of defence is withdrawal. Its antennae are particularly vulnerable to damage, and, as a consequence, they are readily withdrawn when the animal is stimulated. However, there is a

competing requirement for sensory input from both sets of antennae in order for the snail to successfully forage, feed, and reproduce. Habituation of the antennae withdrawal response appears to provide a mechanism by which these competing needs of safety and exposure can be balanced. That is, common, innocuous features of the snails environment are effectively ignored, its antennae remain extended, and necessary sensory information is received.

In many snail containing ecosystems, the animal is often exposed to tactile stimulation, from foliage or water drops for example, and it would be maladaptive if the antennae withdrawal response were to follow every such stimulus. Therefore, it might be expected that the withdrawal response to such stimuli should readily habituate, making the snail an ideal animal for this research programme.

Wherever you go, you will find snails. They are an outstandingly successful member of the gastropod class of molluscs with a foot in virtually every form of ecosystem. Given their ubiquitous nature, there is surprisingly little systematic research on their ability to adapt behaviourally to the multifarious environments in which they are found. This is all the more surprising as habituation, which is one of the important processes of such adaptation, has been studied widely in other gastropods (Harris, 1943; Pakula & Sokolov, 1973).

Much of this work has centred on the lower gastropods, and in particular, the nudibranchiates. Probably the most extensive investigation of habituation in an intact

gastropod was the work of Pieron (1909, 1910, 1911a, 1911b, 1913) in the withdrawal response of marine snails Littorina obtusata and Limnea stagnalis. Individual animals were placed in a strong light and subjected to 15 shadow stimuli each of 1 second duration but with seven ISI conditions (ranging from 3 to 120 secs) as a between-subjects factor. After a rest period of 30 secs, this series was repeated as before. Pieron then plotted the number of shadows necessary for the response to disappear against the ISI. Within this paradigm, the most efficient spacing of shadows for habituation to occur was found to be 1 minute.

Pieron's research also suggests that marine snails benefit by their ability to retain learning to a greater extent than other, 'lower' forms. When the training series of 15 shadow stimuli was administered with an ISI of 1 sec and a variable time period allowed to elapse before repeating the series, a measure of 'savings' for each interseries interval was obtained by dividing the difference between the number of responses from the first and the second series by the number of responses in the first. He reports, with a 2 minute interseries interval, the savings were 77.7%. However, with a full hour between series the savings were still 70.8% (Pieron, 1913).

Further, he used habituation to study the "law of forgetting" by presenting 15 quarter second shadows with an ISI of 10 sec. Snails were retested at post training intervals between 20 sec and 20 hours with a repeated series of stimuli trials. The same measure of savings used in the previous experiment was employed. Pieron was struck by the

resemblance of the resulting curve to that reported by Ebbinghaus for forgetting in humans. Piéron calculated the equation of the curve to be:

$$m = (K (\log t)^{ab}/t)$$

where  $m$  = savings,  $t$  = post training interval, and  $K$ ,  $a$ , and  $b$  are constants (230, 0.5, and 0.36 respectively). He succeeded in describing several curves for different post training interval retests and from this series of experiments arrived at three general rules. First, the more repetitions of the stimuli, the longer an appreciable retention will last. Second, the shorter the ISI, the more repetitions will be necessary to produce an equivalent effect. Third, increasing the ISI longer than about 5 seconds will itself have little effect. These results are of particular interest and will be investigated in the snail Helix aspersa in this thesis (see Chapter 5).

Contraction of the antennae to mechanical stimulation in fresh water snails (eight species of Physa) was studied by Dawson (1911). He investigated this as a function of habitat, and a wide variation in reactivity was reported. This ranged from one animal so sensitive that it responded to air blown onto water in which it lived, to others that only responded to tapping of the antennae themselves. Dawson attributed this to factors such as handling and general disturbance. This attribution was based on his observations comparing a "tamed" snail left undisturbed for a period of weeks with a sensitive snail subjected to maximal handling.

In this experiment, he claims to have demonstrated a complete reversal of behaviour in that the "tamed" snail was demonstrated to be as reactive as the sensitive snail formerly was.

Studies of habituation in terrestrial snails have been limited to the work of Humphrey (1930) and Buytendijk (1921). Humphrey investigated the withdrawal response of the antennae in Helix albolabris to a jerking of the substratum on which it stood (a wooden trolley). Repeated stimulation with an ISI of 2 secs resulted in habituation of the response in 4 to 63 trials, but this was reported to be extremely transient with recovery of the response evident 30 secs post training. Subsequent 30 sec rest periods showed less and less retention.

In another land snail, Limnaeus, withdrawal of the whole exposed part into its shell was found to occur whenever the snail was removed manually from the substratum (Buytendijk, 1921). After a time, it would again extend its anterior portions. Buytendijk observed that if the snail was not roughly handled, the extension took place in less and less time with repeated exposure to the lift stimulus.

The studies reported in this chapter investigate short and long term habituation of the antennae<sup>n</sup> withdrawal response in our most common terrestrial mollusc, Helix aspersa.

#### EXPERIMENT 1

In Helix aspersa, the response to tactile stimulation of the head is not uniform. In the strongest

form of the response, the animal completely withdraws into its shell, whereas, in the weakest form, the antennae remain extended but curl at the end. The purpose of this initial experiment was to evaluate a system of categorising these responses.

Although inter-rater agreement does not necessarily mean that observations are accurate or that interlaboratory agreement will automatically follow (Johnston & Pennypacker, 1980), it would seem a prerequisite of a useful categorizing system that there should be a high degree of inter-rater concordance. Consequently, this experiment reports the agreement between two observers in terms of both response category and response duration.

#### Method

Subjects. Twenty mature snails were selected from a population of 37 which were collected from the Stonehouse area of Plymouth on the night of March 16, 1986. All snails were laboratory housed in a glass vivarium measuring 1 x 1m

Animals were allocated to one of two conditions. In Condition 1, a tactile stimulus was administered, and, in Condition 2, a water drop stimulus was used. Four animals were discarded because of lack of response to the first stimulus or because of experimenter error, and they were replaced from the same population.

Apparatus. The animals were tested on a flat wooden surface which measured 120 x 69 cm. The tactile stimulus was a 200-mm length of 2-mm dowel with rounded

ends. The water stimulus was administered from a Gillette disposable 1-ml syringe and needle. Response durations were recorded with a Smiths 1/10th stopwatch. Illumination was provided by an angle lamp with a 60-W white light bulb.

Procedure. All animals were tested on March 17, 1986, between 08.30 and 11.30 hrs. The temperature in the laboratory was 16 °C.

An active animal was selected from the vivarium and placed on the experimental surface. The experiment commenced 1 min after the animal became active. The tactile stimulus was applied to the head of the snail approximately 2 mm caudal to the dorsal antennae. There were five such stimuli with an ISI of 1 min. The category and duration of the response were recorded by the two experimenters independently of each other. After testing, each animal was returned to a second vivarium and later released into a Plymouth garden. When all the animals in this condition had been tested, the procedure was repeated for animals in Condition 2, except the stimulus was a water drop applied to the head of the animal approximately 2 mm caudal of the dorsal antennae. The single water drop was delivered from a syringe held approximately 0.5 cm above the animal's head.

## RESULTS

Scoring. Since both antennae did not always behave in exactly the same manner, all response measures were taken from the behaviour of the animal's right antenna. (This was used throughout the thesis).

The response was divided into 5 categories. In ascending order of magnitude they were: 0. no change in the animal's behaviour or disposition of its antenna, 1. the end of the antenna curling but no withdrawal of the antenna, 2. partial withdrawal of the antenna, 3. complete withdrawal of the antenna, 4. complete withdrawal of the animal into its shell. For purposes of analysis, the categories were coded as indicated above.

Across categories, the latency of response onset was immeasurably short. Consequently, response onset was taken as occurring at the point of stimulation. The duration of a response was taken from response onset to the reappearance of the animal's eye at the end of the antenna. In the second category of response (antenna curl), the eye did not always withdraw. On these occasions, a response duration of zero was given even though a response was deemed to have taken place. In order that experimenters would not be cued by the click of a watch stopping, the watches were visually inspected while still running in order to obtain the response end point. Consequently, response duration was only measured to the nearest second.

Analysis. Two measures of interobserver agreement were used. First, a percentage agreement score was derived. This was obtained by expressing the number of observations in which observers agreed as a percentage of the total number of observations. Second, the correlation between observers was calculated from all 50 observations in each condition.

In terms of category, in Condition 1, the



percentage agreement score was 84%, and there was a significant correlation between observers ( $\underline{r}(48) = .925$ ,  $p < .001$ ). Experimenter 1 scored the response as larger in 7 of the 8 disagreements. In Condition 2, the percentage agreement score was 88%, and there was a significant correlation between observers ( $\underline{r}(48) = .927$ ,  $p < .001$ ). Experimenter 1 scored the response as larger in all 6 of the disagreements. In both conditions, Category 2 comprised approximately 50% of responses, and the other categories made an approximately equal contribution to the remainder. In neither condition, was disagreement between observers greater than one category.

In terms of duration, in Condition 1, the percentage agreement score was 82%, and there was a significant correlation between observers ( $\underline{r}(48) = .970$ ,  $p < .001$ ). Experimenter 1 scored the duration as longer in 7 of the 9 disagreements. The discrepancy was greater than 1 sec in only one case. The range of durations was from 0 to 25 sec with a mean of 7.10 sec and standard deviation of 6.760 for Observer 1, whereas there was a range of 0 to 25 sec with a mean of 7.00 sec and standard deviation of 6.691 for Observer 2. In Condition 2, the percentage agreement was 78%, and there was a significant correlation between observers ( $\underline{r}(48) = .947$ ,  $p < .001$ ). Experimenter 1 scored the duration as longer in 7 of the 11 disagreements. The discrepancy was never greater than 1 sec. The range of durations was from 0 to 45 sec with a mean of 6.56 sec and standard deviation of 8.987 for Observer 1, whereas there was a range of 0 to 45 sec with a mean of 6.5 sec and a

standard deviation of 9.043 for Observer 2.

### Discussion

The results of Experiment 1 show a high degree of inter-observer agreement in terms of both category and duration of response. Consequently, these measures were used throughout the experiments which follow. Nevertheless, agreement was not 100%, and this points to the need for considerable practice in scoring responses before embarking on experimentation in this area.

### EXPERIMENT 2

The first requirement for a demonstration of habituation is that the response should decrease across iterated presentations of a stimulus. The experiment reported here is one of many carried out in my laboratory in which such a decrement has been observed.

### METHOD

Subjects. Mature snails were captured from a Plymouth garden in wet conditions on February 18, 1986. Data were collected from 10 snails. Two were discarded during the experiment because they reared at the time of stimulus presentation, and they were replaced from the same population. Animals were housed in the same conditions as in Experiment 1.

Apparatus. The apparatus was the same as that reported in Experiment 1, except that only the tactile stimulus was employed.

Procedure. The experiment took place on February 19, 1986, between 24.00 and 02.00 hrs. There was low cloud throughout the experiment with a mean relative humidity of 72% and mean temperature of 3 C. The temperature in the laboratory was 18 C.

Each animal was placed on the experimental area 60 sec prior to the commencement of training. Presentation of the tactile stimulus was the same as that in Experiment 1. Each animal received 10 presentations of the tactile stimulus with 30-sec ISIs. Unlike Experiment 1, the watch was stopped when each response was terminated. Otherwise, the recording of responses was the same as in Experiment 1.

## Results

Both response measures were analysed in one-way, repeated measures ANOVAs. In this, and all other repeated measures analyses reported, conservative degrees of freedom were employed (Greenhouse & Geisser, 1959). For both response duration and category, there was a significant decline across repeated presentations of the stimulus ( $F(1/9) = 59.90, p < .001$  and  $66.64, p < .001$  respectively) (see Figure 3.1). In terms of response duration, there was a significant difference between the mean response to the first stimulus (20.20 sec) and that to the last (0.20 sec) ( $F(1/9) = 265.745, p < .001$ ). The same was true in terms of

mean response category to the first (3.7) and last stimuli (0.2) of the series ( $F(1/9) = 313.941, p < .001$ ). The decrement in response is illustrated for response duration in Figure 3.1, and magnitude Figure 3.2.

It is commonly observed with habituation data (e.g. Lader, 1969) that, when mean response magnitude is plotted against log stimulus number, rectilinearity is achieved. When this was done for response duration, there was significant linearity ( $r(8) = -0.991, p < .001$ ), and the expected negative slope was obtained (-20.910). Similarly, with response category, there was significant linearity ( $r(8) = -0.938, p < .001$ ), and, again, a negative slope was obtained (-3.362).

#### DISCUSSION

The expected decrement across trials was obtained, and it conformed in shape to that typical of habituation data.

#### EXPERIMENT 3

It proved impossible to automate stimulus administration without drastically affecting the general behaviour of the snail. Consequently, the stimulus had to be administered by hand. There is, therefore, the possibility that any demonstration of response decrement across trials might be contaminated by experimenter effects. In order to provide a demonstration of response decrement across trials

FIGURE 3.1

DECREMENT IN RESPONSE DURATION WITH REPEATED PRESENTATIONS OF STIMULUS

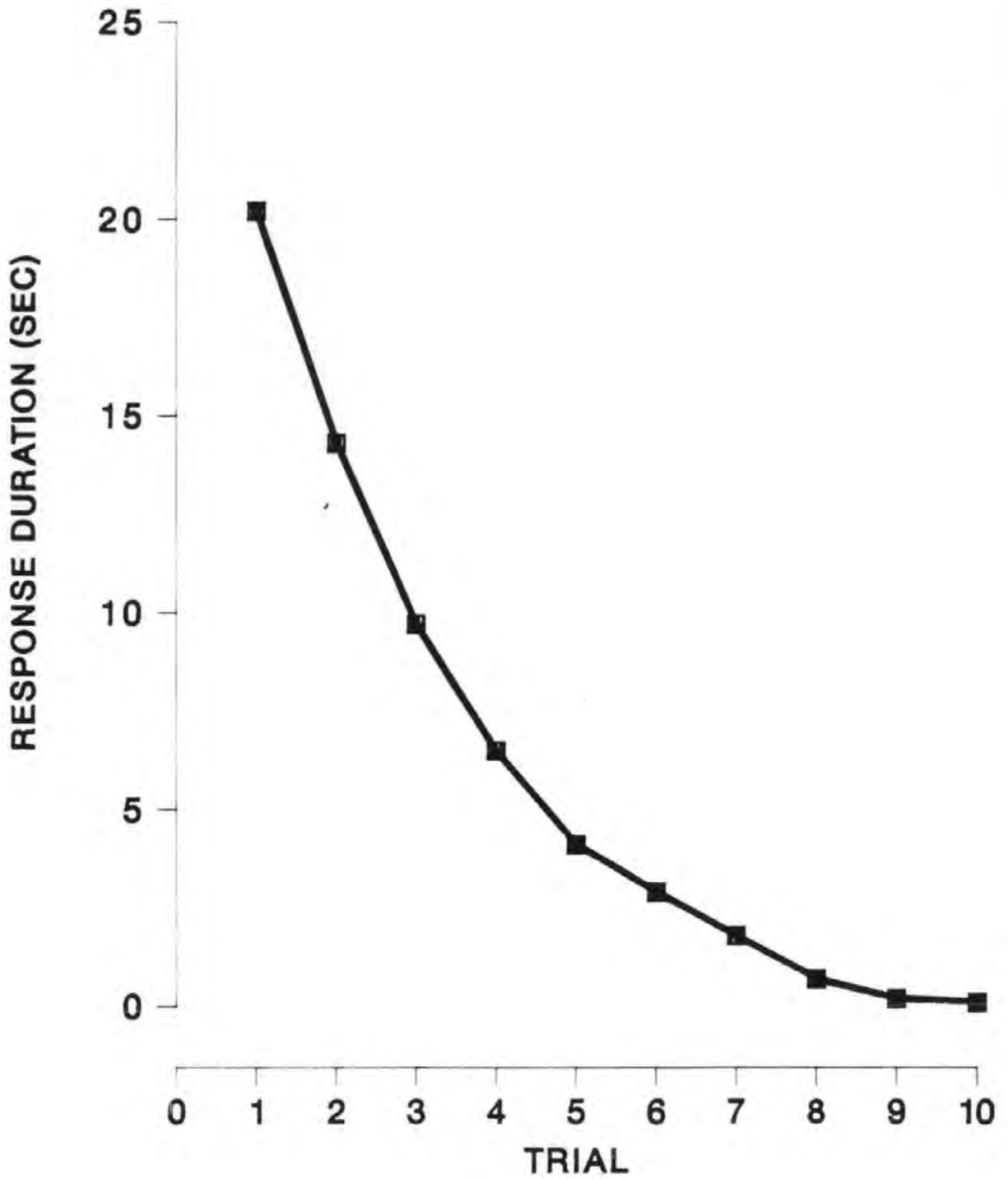
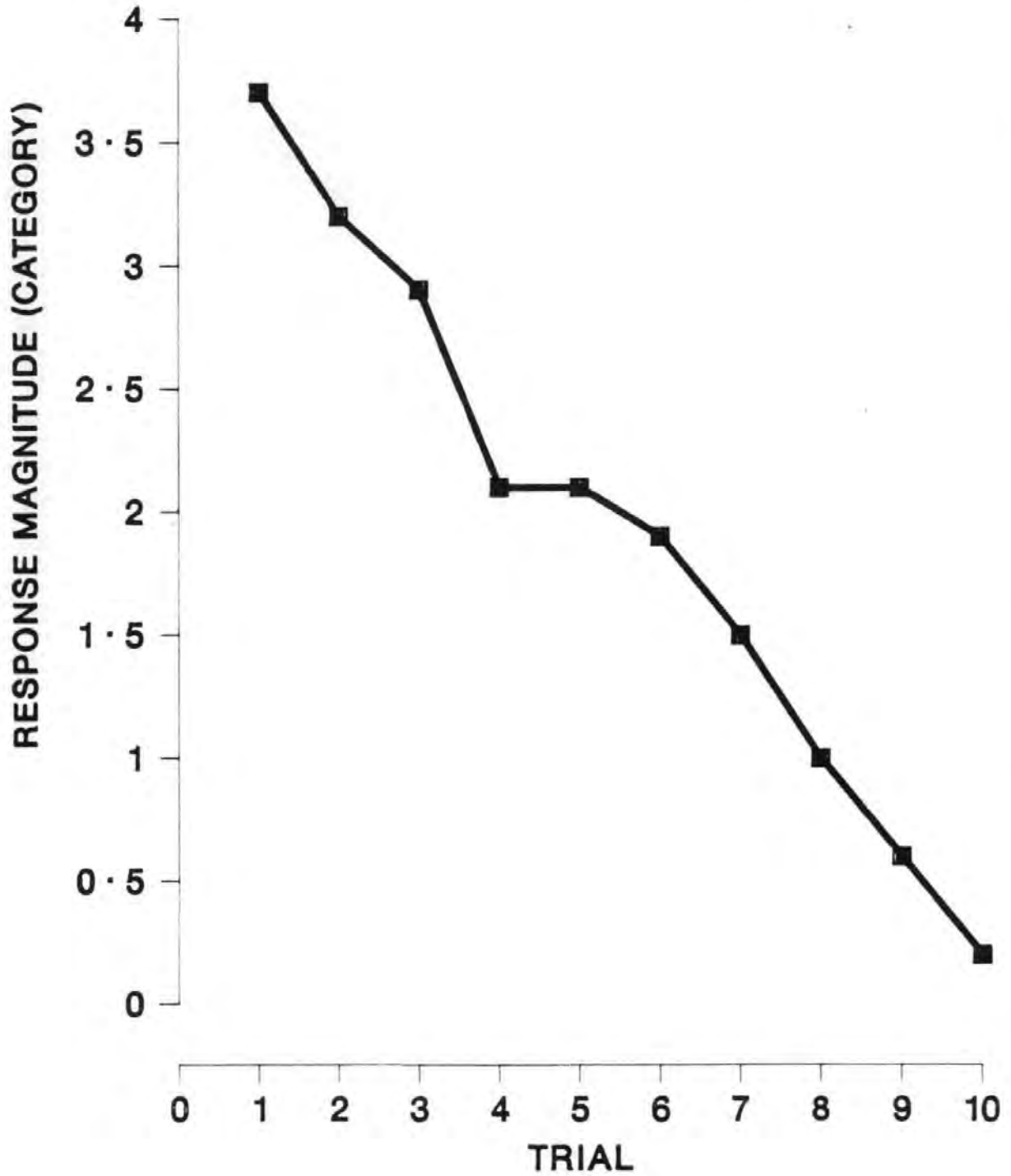


FIGURE 3.2

DECREMENT IN RESPONSE MAGNITUDE WITH REPEATED PRESENTATIONS OF STIMULUS



which was not contaminated by such effects, the following double-blind experiment was carried out.

### Method

Subjects. Eighty adult snails collected from the Bovisand area of Plymouth on June 14 and 17, 1986, were used in this experiment. They were allocated to one of eight conditions. Six snails were discarded due to rearing during stimulus administration or permanent withdrawal into their shell. These were replaced by snails from the same population.

Apparatus. Animals were tested on a flat, white melamine surface which measured 60 x 55 cm. The tactile stimulus was an 150-mm long aluminium prod with a rounded end of 4-mm diameter. Illumination was provided by an overhead, 60-W, white light bulb. Response durations were measured using a Smiths 1/10th stopwatch.

Procedure. The experiment took place between midnight and 06.00 hrs on June 23 and 24, 1986. The temperature in the laboratory varied between 20 and 21 C.

Animals received one of eight, pre-training conditions which consisted of 0, 1, 2, 3, 4, 5, 6, or 7 presentations of the tactile stimulus applied approximately 2 mm caudal to the dorsal antennae. Experimenter 1 removed the snail from the home vivarium 30 sec before the first trial of pretraining or 30 sec before the test stimulus if the animal was in the 0 pretraining condition. The order of conditions was randomly determined, and it was not known to

Experimenter 2 who was called to administer the test stimulus 30 sec after the last stimulus of pretraining. This test stimulus was the same as the stimuli of pretraining and was applied in the same manner. The response to the test stimulus was recorded by Experimenter 2, and, depending upon which condition the snail was in, this response was counted as Trial 1, 2, 3, 4, 5, 6, 7, or 8 in an habituation series. In order that no time cues were available to Experimenter 2, he was called to present the test stimulus at 5-min intervals which allowed time for the most extensive of the pretraining conditions to be carried out. During pretraining, Experimenter 2 sat approximately 4 m away from the experimental area with his back to it, while he watched videos. Thus, Experimenter 2 had no knowledge of which condition each animal was in and, consequently, which stimulus in the habituation series he was presenting.

## RESULTS

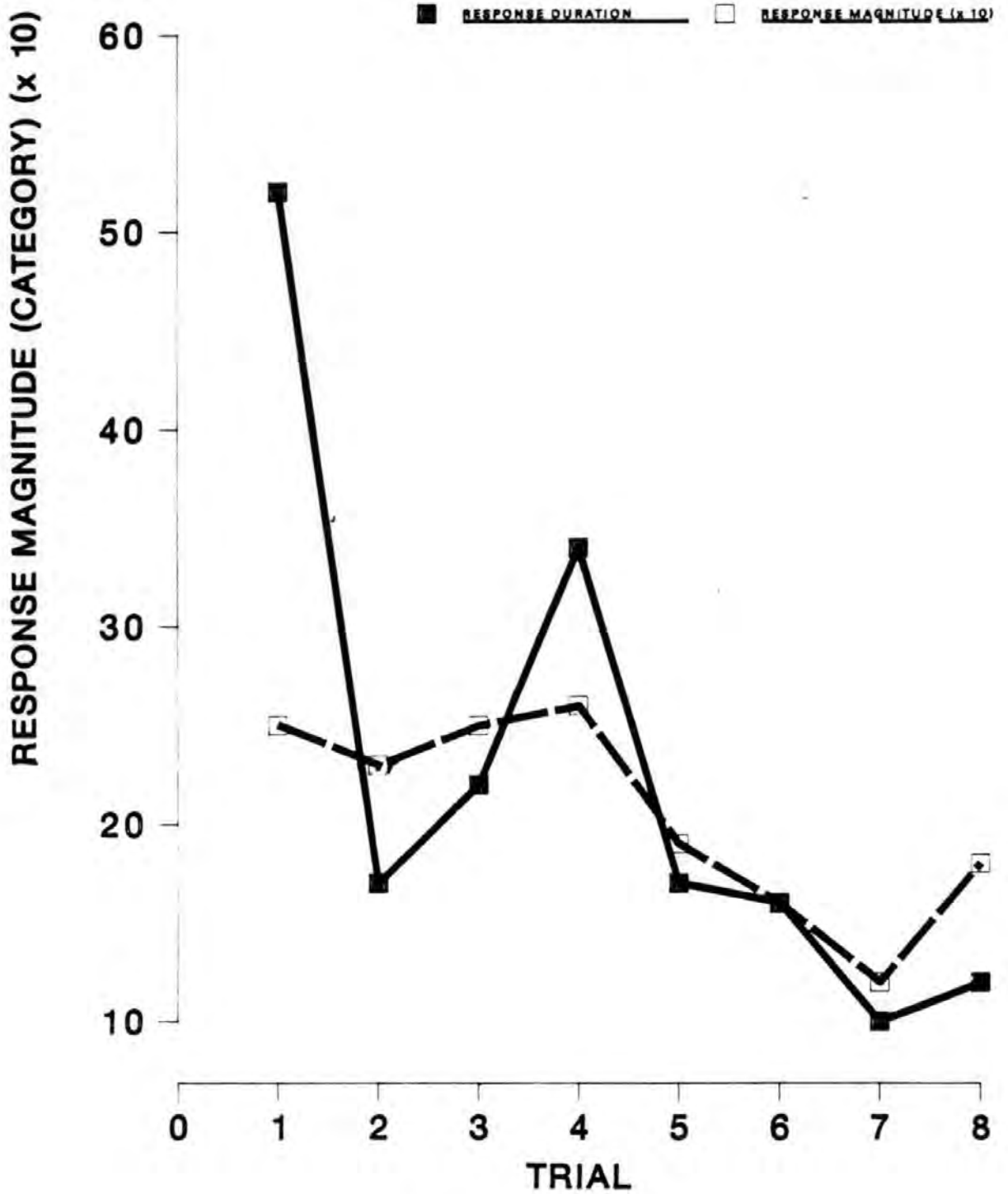
The responses to the test stimuli were scored in terms of category of response magnitude and response duration in the same manner as that employed in Experiment 1.

There was a decline in response across trials both in terms of duration and response category. However, this decline was more pronounced for duration than category (see Figure 3.3). This was born out in a between subjects ANOVA in which the decline across trials was significant for duration ( $F(7/72) = 2.20, p < .05$ ) but not quite for category



FIGURE 3.3

A COMPARISON BETWEEN DECREMENT OVER 8 TRIALS  
BETWEEN RESPONSE DURATION AND MAGNITUDE SCORES



( $F(7/72) = 1.98, p > .05$ ). The difference between first and last trial was significant for duration ( $F(1/72) = 9.03, p < .01$ ) but was not significant for category using a Newman-Keuls test ( $q(5/72) = 2.20, p > .05$ ). However, both sets of data produced significant rectilinearity when mean response magnitude was plotted against log stimulus number (for duration,  $r(6) = -.794, p < .05$  and, for category,  $r(6) = .719, p < .05$ ), and both displayed the expected negative slope (for duration,  $-3.725$  and, for category,  $-.907$ ).

## DISCUSSION

There was good evidence for the expected decline across trials in terms of response duration, but it was less convincing in terms of category. Although it was apparent, the trials effect was not as strong as we find in the usual habituation experiment, but, given the design of this experiment, this was to be expected. The design employed to eliminate any possible experimenter effect also militated against a trials effect in two ways. First, the test trial was administered by a different experimenter to the one who administered the pretraining trials and, to some extent, would therefore constitute a changed stimulus. Second, habituation experiments use within subject designs when looking at trials effects, and these are much more sensitive than the between subject design used for trials in this experiment. Nevertheless, despite these factors, a trials effect was evidenced.

## EXPERIMENT 4

Demonstration of a decline in response across trials does not in itself constitute sufficient evidence for habituation, as there are other phenomena which might produce such a decrement (e.g. fatigue). One of the main ways in which habituation is differentiated from such phenomena is through the change stimulus effect. Indeed, one of the parametric characteristics of habituation proposed by Groves and Thompson (1970) is that the habituated response should return when the stimulus is changed. The series of experiments which follow were carried out in order to investigate the effects of a change in stimulation after habituation of the antennae withdrawal response in the snail.

### Experiment 4a

Subjects. Adult snails collected on October 22, 1984, from the Bovisands area of Plymouth were employed in this experiment. They were housed in a glass, laboratory vivarium which measured 1 x 1 m. Forty animals were allocated randomly to one of four groups. Six snails were discarded because they did not respond or were rearing at the time of stimulus presentation, and they were replaced from the same population.

Apparatus. The tactile stimulus was a 200-mm length of 2-mm dowel with rounded edges. The water drop stimulus was applied from a Gillette disposable 1-ml syringe

and needle. The experiment took place on a white melamine board which measured 60 x 55 cm. Illumination was provided by an overhead, 60-W, white light bulb. Response duration was recorded with a Smith's 1/10th sec stop watch.

Procedure. The experiment took place on October 23, 29, and 30, 1984, between 18.30 and 21.30 hrs. The temperature in the laboratory was between 19 and 21 C.

There were four conditions. In Condition 1, animals were trained with the tactile stimulus until they reached the habituation criterion of three consecutive non responses. They were then presented with the water drop stimulus which served as a test stimulus. In Condition 2, snails were trained to criterion with the water drop stimulus and then given one further water drop, test stimulus. In Condition 3, they were trained to criterion with the water drop stimulus and then presented with the tactile, test stimulus. In Condition 4, the snails were trained to criterion with the tactile stimulus and then given one more presentation of the tactile stimulus as a test. Consequently, Conditions 1 and 3 comprised the change conditions and 2 and 4 the no change conditions.

Snails were allocated to conditions by a third experimenter. Each snail was removed from its home vivarium and placed on the experimental area 60 sec before the start of training. The appropriate stimulus was then applied at 30-sec intervals until the criterion of habituation had been reached. The second experimenter then applied the appropriate post training, test stimulus. This experimenter did not observe training, and therefore, did not know to

which condition the animal was allocated. The duration and category of the response to the test stimulus was recorded by the second experimenter.

## RESULTS

All results were analysed in a one-way ANOVA. There was a significant difference between groups in terms of duration of response to the test stimulus ( $F(3/36) = 31.03$ ,  $p < .001$ ). Further analysis indicated that there was a significant difference between the means of the change (25.15 sec) and non change (1.8 sec) conditions ( $F(1/36) = 63.828$ ,  $p < .001$ ). Further comparison indicated that the largest effect came when animals in Condition 1 ( $\bar{M} = 36.4$  sec) were compared with those in Condition 2 ( $\bar{M} = 1.5$  sec) ( $F(1/36) = 70.895$ ,  $p < .001$ ). The difference between those in Condition 3 ( $\bar{M} = 13.9$  sec) and those in Condition 4 ( $\bar{M} = 2.1$  sec) was smaller but still significant ( $F(1/36) = 8.286$ ,  $p < .01$ ).

There was also a significant difference between groups in terms of category of response to the test stimulus ( $F(3/36) = 25.51$ ,  $p < .001$ ). Further analysis indicated that there was a significant difference between the means of the change (3.15) and non change (0.95) conditions ( $F(1/36) = 66.484$ ,  $p < .001$ ). Again, further comparison indicated that the largest effect came between Condition 1 ( $\bar{M} = 3.7$ ) and Condition 2 ( $\bar{M} = 0.7$ ) ( $F(1/36) = 61.810$ ,  $P < .001$ ). The difference between those in Condition 3 ( $\bar{M} = 2.6$ ) and those in Condition 4 ( $\bar{M} = 1.2$ ) was smaller but still significant.

( $F(1/36) = 13.462, p < .001$ ).

## DISCUSSION

As predicted, the presentation of a change stimulus resulted in an increase in both duration and category of response. Further, with both measures, the change from tactile to water drop stimulus seemed to provoke a larger response than that from water drop to tactile stimulus.

As well as this return of the response to a change stimulus, Groves and Thompson (1970) have argued that an habituated response should display dishabituation. That is, the response to the training stimulus should return after interpolation of a change stimulus. In order to replicate the above results and demonstrate dishabituation, a second experiment was carried out using the same stimuli.

### Experiment 4b

#### Method

Subjects. Mature snails were collected from a garden in central Plymouth, in wet conditions, on the night of October 14, 1985. The laboratory housing was the same as that in Experiment 4a. Forty animals were randomly allocated to the four conditions. Five snails were discarded and replaced from the same population.

Apparatus. The apparatus was the same as that used in Experiment 4a.

Procedure. The experiment was conducted on the nights of October 15 and 17, 1985, between 18.00 and 21.30 hrs. The temperature in the laboratory ranged from 19 to 22 C. The procedure was the same as that used in Experiment 4a except that an additional training stimulus (second test stimulus) was administered after the first test stimulus had been presented. Neither experimenter knew to which condition an animal belonged. Animals were allocated to conditions by a third experimenter. Experimenter 1 trained the snails and administered the second test stimulus. For half the snails, this would be a return to the training stimulus after a change stimulus (dishabituation), and, for the other half, it would merely be a further below zero training trial. Experimenter 2 administered the first test stimulus. Neither experimenter was present when the other was stimulating the snails.

The four conditions were as follows: Condition 1 in which the training stimulus was tactile and was followed by a water drop as the change stimulus with one further presentation of the tactile stimulus, Condition 2 in which the water drop was used throughout the experiment, Condition 3 in which the training stimulus was a water drop followed by the tactile stimulus as a change stimulus and a further presentation of the water drop, Condition 4 in which the tactile stimulus was used throughout.

### Results

In terms of response duration, there was a

significant difference between groups in response to the first test stimulus ( $F(3/36) = 3.56, p < .05$ ). A planned comparison indicated that the response to the change stimulus ( $M = 11.35$  sec) was significantly larger than that to the equivalent stimulus in the control condition ( $M = 5.1$  sec) ( $F(1/36) = 6.673, p < .05$ ). However, further planned comparisons revealed that the difference between the Condition 3 ( $M = 10.3$  sec) and Condition 4 ( $M = 1.9$  sec) was significant ( $F(1/36) = 5.603, p < .05$ ), whereas the difference between Condition 1 ( $M = 12.7$  sec) and Condition 2 ( $M = 8.3$  sec) was not significant ( $F(1/36) = 1.654, p > .05$ ).

In terms of response category, a similar pattern emerged. There was a significant difference between groups ( $F(3/36) = 9.00, p < .001$ ). Further analysis revealed a difference between the means of the change (2.1) and non change (1.25) conditions ( $F(1/36) = 17.00, p < .001$ ). However, although the difference between the means of Condition 3 (2.0) and Condition 4 (0.8) was significant ( $F(1/36) = 16.941, p < .001$ ), the difference between those of Condition 1 (2.2) and Condition 2 (1.7) was not significant ( $F(1/36) = 2.941, p > .05$ ).

The results for the test of dishabituation were somewhat more uniform. In terms of response duration, there was a significant difference between groups ( $F(3/36) = 5.710, p < .01$ ). The planned comparison revealed a significant difference ( $F(1/36) = 16.027, p < .001$ ) between the means of the dishabituation groups (14.7 sec) and the non change groups (5.25 sec). Further analysis revealed a significant difference between Condition 1 ( $M = 13.3$  sec)



and Condition 2 ( $\underline{M} = 4.6$  sec) ( $\underline{F}(1/36) = 4.427$ ,  $p < .05$ ). Similarly, there was a significant difference ( $\underline{F}(1/36) = 12.965$ ,  $P < .001$ ) between Condition 3 ( $\underline{M} = 16.1$  sec) and Condition 4 ( $\underline{M} = 0.9$  sec).

Again, for response category, there was a significant difference between groups ( $\underline{F}(3/36) = 9.09$ ,  $p < .001$ ). The planned comparison demonstrated a significant difference ( $\underline{F}(1/36) = 25.039$ ,  $p < .001$ ) between the means of the dishabituation groups (2.45) and the non change groups (0.65). Further analysis revealed a significant difference ( $\underline{F}(1/36) = 6.530$ ,  $p < .05$ ) between the means of Condition 1 (2.3) and Condition 2 (1.0). There was a larger significant difference ( $\underline{F}(1/36) = 20.440$ ,  $p < .001$ ) between the means of Condition 3 (2.6) and Condition 4 (0.3).

## DISCUSSION

Although these results again demonstrated the return of the response to a change stimulus, they were not as clear cut as those from Experiment 4b. Indeed, to some extent, they contradicted those results. In Experiment 4b, the water drop proved to be a more effective change stimulus than did the tactile stimulus. In this experiment, the reverse was the case to the extent that, in terms of response duration, there was not a significant increase in response to the water drop, change stimulus.

Somewhat to my surprise, the results for dishabituation were stronger than those for the change stimulus. Although the water drop change stimulus was less

effective than the tactile stimulus in provoking dishabituation, both provided clear evidence that an interpolated change stimulus results in a return of the antennae withdrawal response to a previously habituated stimulus.

The reversal of the potency of the two stimuli in terms of their effect as a change stimulus was curious. Therefore, I decided to carry out a further Change Stimulus Experiment with these stimuli.

#### Experiment 4c

##### Method

Subjects. Mature snails were collected from the Stonehouse area of Plymouth on the night of May 14, 1986, in dry conditions. They were then housed in the same conditions as in Experiment 4a. Forty animals were randomly allocated to the four conditions. Five snails were discarded and replaced from the same population.

Apparatus and Procedure. The apparatus, procedure, and experimental conditions were the same as those employed in Experiment 4a.

The experiment was run between 23.30 hrs on May 15 and 06.15 hrs on May 16, 1986. The mean laboratory temperature was 19 C.

## RESULTS

In terms of duration of the response to the test stimulus, there was a significant difference between groups ( $F(3/36) = 23.250, p < .001$ ). The planned comparison revealed a significant difference ( $F(1/36) = 48.188, p < .001$ ) between the means of the change stimulus groups (26.6 sec) and the non change groups (1.6 sec). Further analysis showed that animals in Condition 1 ( $M = 38.4$  sec) displayed significantly larger responses than those in Condition 2 ( $M = 1.3$  sec) ( $F(1/36) = 53.061, p < .001$ ), and, although the difference was smaller, those in Condition 3 ( $M = 14.8$  sec) displayed significantly larger response than those in Condition 4 ( $M = 1.9$  sec) ( $F(1/36) = 12.965, p < .01$ ).

For the category data, there was a significant difference between groups ( $F(3/36) = 27.58, p < .001$ ), and the planned comparison revealed a significant difference ( $F(1/36) = 70.903, p < .001$ ) between the means of the change groups (3.2) and the non change groups (0.95). Further analysis showed that animals in Condition 1 ( $M = 3.8$ ) displayed significantly larger responses than those in Condition 2 ( $M = 0.7$ ) ( $F(1/36) = 67.294, p < .001$ ), and those in Condition 3 ( $M = 2.6$ ) displayed significantly larger responses than those in Condition 4 ( $M = 1.2$ ) ( $F(1/36) = 13.726, p < .01$ ).

## DISCUSSION

Again, there was clear evidence that the antennae

withdrawal response returns to a change stimulus. As in Experiment 4a, the water drop appeared to provoke a greater return of the response than did the tactile stimulus.

In order to extend the range of these findings, it was decided to carry out a further change stimulus experiment using a stimulus other than the water drop.

#### Experiment 4d

##### Method

Subjects. Mature snails were collected from Devil's Point in Plymouth on June 16, 1986, in dry conditions. Forty animals were randomly allocated to one of the four conditions. Four snails were discarded and replaced from the same population.

Apparatus. The apparatus was the same as that for Experiment 4a except that the stimuli were a 150-mm long aluminium prod with a rounded end of 4-mm diameter and a 'Woolworth's' fine art brush with bristles trimmed back to 2 mm. It was thought that snails may be able to detect the difference between the smooth surface of the aluminium prod and the rough surface of the brush.

Procedure. The procedure was the same as that employed in Experiment 4a. The brush stimulus was presented so that the ends of the bristles were tapped against the animals head in the same way as the tactile stimulus in Experiment 4a.

The four conditions were as follows: Condition 1

in which the animal was trained with the prod and had the brush as a change stimulus, Condition 2 in which the stimulus was the brush throughout, Condition 3 in which the animal was trained with the brush and the prod was the change stimulus, Condition 4 in which the stimulus was the prod throughout.

The experiment was carried out on June 18, 1986, between 00.15 and 05.50 hrs. The temperature in the laboratory ranged from 18 to 20 C.

## RESULTS

In terms of duration of the response to the test stimulus, there was not an overall significant difference between groups ( $F(3/36) = 2.07, p > .05$ ). Although the mean response of the change groups (3.75 sec) was larger than that of the non change groups (1.72 sec) in the planned comparison, this difference was not significant ( $F(1/36) = 2.842, p > .05$ ). Further analysis revealed no significant difference ( $F(1/36) < 1$ ) between the means of Condition 1 (4.10 sec) and Condition 2 (3.25 sec). Animals in Condition 3 ( $\bar{M} = 3.40$  sec) did produce larger responses than those in Condition 4 ( $\bar{M} = 0.20$  sec), but this difference was not quite significant ( $F(1/36) = 3.548, p > .05$ ).

In terms of response category, there was an overall significant difference between groups ( $F(3/36) = 3.300, p < .05$ ). The planned comparison again revealed no significant difference between the change groups ( $\bar{M} = 1.25$ ) and the non change groups ( $\bar{M} = 0.8$ ) ( $F(1/36) = 2.163, p > .05$ ). Although

further analysis again revealed no significant difference ( $F(1/36) < 1$ ) between the means of Condition 1 (1.30) and Condition 2 (1.40), the mean responses in Condition 3 (1.20) were significantly larger ( $F(1/36) = 5.341, p < .05$ ) than those in Condition 4 (0.20).

## DISCUSSION

The results did not provide convincing evidence of a change stimulus effect with these two stimuli. This may have been because the animal cannot discriminate between the two stimuli, but, on the other hand, the results were generally in the direction predicted. Consequently, it was decided to carry out a further change stimulus experiment with these stimuli and to include an investigation of dishabituation.

### Experiment 4e

#### Method

Subjects. Mature snails were collected from a Plymouth railway embankment in wet conditions on June 18, 1986. Forty snails were randomly allocated to one of the four conditions. Six snails were discarded and replaced from the same population.

Apparatus and Procedure. The apparatus was the same as that employed in Experiment 4d, and the procedure was the same as that of Experiment 4b with the brush

replacing the water drop stimulus.

The experiment was conducted on June 19, 1986, between 01.30 and 06.45 hrs. The temperature in the laboratory ranged from 19 to 21 C.

## RESULTS

In terms of the response to the first test stimulus, there was no overall difference between groups in response duration ( $F(3/36) = 1.170, p > .05$ ). The change group did give longer responses ( $M = 4.28$  sec) than the non change group ( $M = 1.80$  sec), but this was not significant ( $F(1/36) = 2.772, p > .05$ ). Again, further analysis did not reveal a significant difference ( $F(1/36) = 1.47, p > .05$ ) between the means of Condition 1 (4.95 sec) and Condition 2 (2.4 sec), and nor was there a significant difference ( $F(1/36) = 1.300, p > .05$ ) between the means of Condition 3 (3.60 sec) and Condition 4 (1.20 sec).

There was no overall difference between groups in terms of category of response to the first test stimulus ( $F(3/36) = 1.44, p > .05$ ). However, the planned comparison revealed a significant difference ( $F(1/36) = 4.118, p < .05$ ) between the means of the change (1.55) and non change groups (0.85). Nevertheless, in further analysis, the difference between Condition 1 ( $M = 1.50$ ) and Condition 2 ( $M = 0.80$ ) was not significant ( $F(1/36) = 2.059, p > .05$ ) and nor was the difference between Condition 3 ( $M = 1.60$ ) and Condition 4 ( $M = 0.90$ ) ( $F(1/36) = 2.059, p > .05$ ).

The effects of dishabituation were, however, more

pronounced. In terms of response duration, there was a significant overall difference between groups ( $F(3/36) = 8.240, p < .001$ ). The planned comparison revealed that the mean response to the test stimulus presented after a change stimulus (dishabituation) was significantly longer (5.85 sec) than that to the test stimulus presented after the equivalent non change stimulus (0.48 sec) ( $F(1/36) = 22.966, p < .001$ ). Further analysis revealed that there was a significant difference ( $F(1/36) = 7.349, p < .05$ ) between the means of Condition 1 (4.80 sec) and Condition 2 (0.50 sec) and a larger significant difference ( $F(1/36) = 16.535, p < .001$ ) between those of Condition 3 (6.90 sec) and Condition 4 (0.45 sec).

In terms of response category, the same pattern emerged. There was an overall significant difference between groups ( $F(3, 36) = 23.000, p < .001$ ), and the response to the dishabituation stimulus ( $M = 2.10$ ) was significantly larger than that to the equivalent stimulus in the non change groups ( $M = 0.40$ ) ( $F(1, 36) = 24.764, p < .001$ ). Further planned comparisons revealed that the mean response in Condition 1 (1.90) was significantly larger than that in Condition 2 (0.5) ( $F(1, 36) = 8.398, p < .01$ ), and, in Condition 3 (2.30), it was significantly larger than that in Condition 4 (0.3) ( $F(1, 36) = 17.138, p < .001$ ).

## DISCUSSION

Again, although there was some indication that the response did return to a change in stimulation with these



two stimuli, it was not a very strong effect. However, dishabituation was unequivocally demonstrated, and this would imply that the animals could discriminate between the two stimuli.

#### GENERAL DISCUSSION

The experiments reported in this chapter have demonstrated habituation of the antennae withdrawal response in the snail in terms of a response decrement across trials, a return of the response to a change stimulus, dishabituation, and retention of habituation.

The adaptive value of habituation of this response for the snail is easy to see. Similarly, the return of the response to a change stimulus has adaptive value, as any change in the environment may signal a new, potential threat to the important but delicate sensory systems of the antennae.

It is, however, difficult to see an adaptive function for the large dishabituation effect relative to that produced by the change stimulus itself. It may be the result of a delayed sensitization effect produced by a change in stimulation. This interpretation is in accord with the account of dishabituation put forward in the Dual-Process theory (Groves & Thompson, 1970).

Investigation of this form of learning in the snail does have its drawbacks. For example, the snail is most active at night, and its activity varies from season to season. However, not only is this form of adaptive behaviour

of interest in itself, but the snail's relatively simple nervous system makes habituation of this response extremely conducive to an investigation of the underlying processes of habituation, and in particular the effects of protein synthesis inhibition.

Before this can be done with much confidence, however, it is important to further establish this habituation as true learning by demonstrating long term retention of the habituation and investigating the parameters of such retention.

## CHAPTER 4

### LONG-TERM HABITUATION IN THE SNAIL HELIX ASPERSA, RETENTION AND RECOVERY FROM ONE TRAINING SESSION.

In the preceding chapter, a suitable methodology has been established for the study of the antennae withdrawal response in the snail Helix aspersa. This response is demonstrated to decrement with iterated presentations of a stimulus. That this decrement is habituation is demonstrated by a series of change stimulus experiments.

A definitive characteristic of habituation is that if the stimulus is withheld, the response recovers over time (Harris, 1943). Perhaps above all habituation is demonstrated as a form of true learning by its long term retention (see Chapter 2).

Recovery of a response after habituation has been studied utilising a variety of measures with the relative completeness of recovery remaining a source of debate in the literature. Also posited has been the suggestion that phylogenetic position is a determinant of recovery (Harris, 1943; Humphrey, 1933). These authors suggest that, in simpler organisms, and simple responses in higher organisms, long term habituation occurs only after a very large number of stimulus presentations.

Reported, are two experiments that sample recovery of the antennae withdrawal reflex from habituation measured by number of savings in trials required to reach a criterion of habituation at retest and recovery of initial response level

at retest.

## EXPERIMENT 1

An initial experiment was conducted to investigate the time course of recovery of the response in the snail Helix aspersa immediately after training. Response recovery was measured by comparison of the initial response duration and magnitude in training with the first response at each of ten post-training retests. The response was said to have recovered when there was no difference between these two responses.

### Method

Subjects. Fifty mature snails were collected from a wooded valley site in Jennycliff Bay, South Devon. The snails were laboratory housed in a glass vivarium which contained damp vegetation.

Procedure. Habituation trials commenced 24 hrs after capture and were conducted between 2000 and 0845 hrs on the nights of April 15, 16, 17, 1985, under subdued lighting provided by a 60-w red bulb in an angle poise lamp situated above the test apparatus. Temperature in the laboratory varied from 19 to 21 C. Training consisted of the snail being lifted from the vivarium and placed on a pinewood dissecting board 80 x 45 cm. The animal remained on the test board unmolested for one minute. If the snail did not emerge from its shell during this period, its participation in the

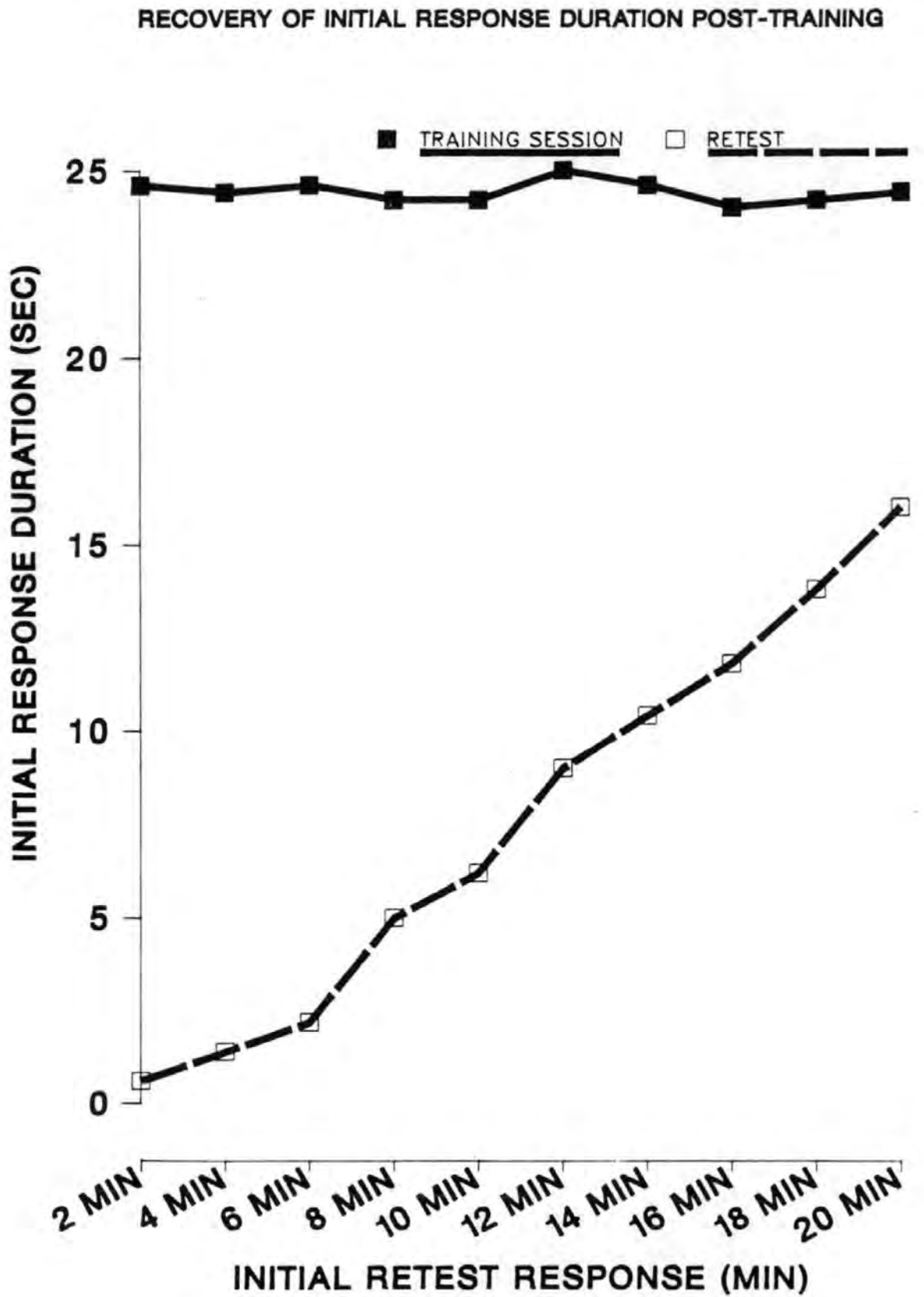
experiment was terminated. Four such substitutions were necessary. Fully extended snails received repeated presentations of a tactile stimulus with an ISI of 30 sec. The tactile stimulus was a touch approximately 2 mm caudal to the dorsal antennae from a 35 mm length of 2-mm dowel. Repeated presentations continued until they reached criterion of habituation. Criterion for habituation was three consecutive non responses. Trials to habituation were recorded exclusive of these three non responses. This criterion of habituation was used throughout the thesis unless otherwise stated. Five snails were sampled for recovery of the response at each of the following post training intervals: 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 min. All responses were measured for duration and magnitude as reported in Experiment 1, Chapter 3.

## RESULTS

Recovery of the response post-training is shown in Figure 4.1. Duration of response data were analysed in a 2-way ANOVA with a within subject factor of trial (first response training/first response at retest) and a between subjects factor of post-training retest interval. This revealed a significant effect of post-training interval ( $F(9/40) = 6.287, p < .01$ ), and of trial ( $F(9/40) = 640.115, p < .001$ ) with a significant post training interval x trial interaction ( $F(9/40) = 6.794, p < .01$ ).

Analysis of simple main effects revealed no difference between groups in terms of initial response in training

FIGURE 4.1



( $F(1/40) < 1$ ) but a significant difference in initial response between retest conditions ( $F(1/40) = 352.52$ ,  $p < .001$ ).

Within group comparisons of initial training and retest responses showed the initial response in retest to be significantly smaller than the initial response evidenced in training in all ten retest conditions.

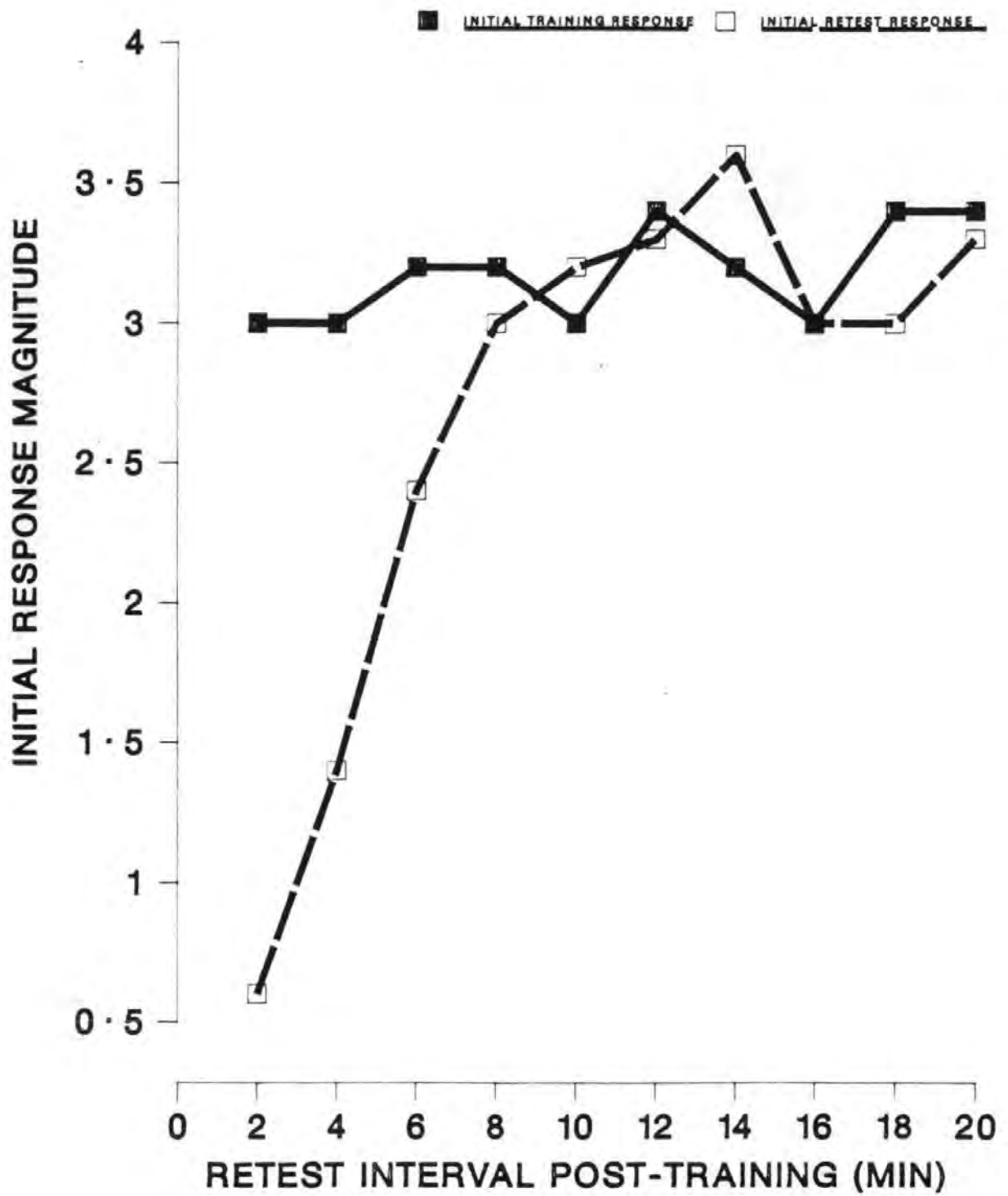
As can be seen from Figure 4.1, initial response at retest increased as a function of elapsed time from training. The response had not totally recovered by the maximum post-training interval sampled.

Magnitude of response data was also similarly analysed and revealed a significant effect of post-training interval ( $F(1/40) = 12.69$ ,  $p < .001$ ) and of trial ( $F(1/40) = 25.20$ ,  $p < .001$ ) with a significant post-training interval x trial interaction ( $F(1/40) = 12.12$ ,  $p < .025$ ).

Analysis of simple main effects showed that in comparisons between groups there was no difference in initial training response ( $F(1/40) = 2.80$ ,  $p > .05$ ), but there was a significant difference in the magnitude of the first response at retest between groups ( $F(1/40) = 512.23$ ,  $p < .001$ ). Comparisons within groups across training and retest response revealed that the response had recovered to its initial magnitude by the 8 min retest ( $F(1/40) < 1$ ). The results for this measure are shown in Figure 4.2.

FIGURE 4.2

RECOVERY OF INITIAL RESPONSE MAGNITUDE AT RETESTS





## DISCUSSION

The above results suggest that habituation of the dorsal antennae withdrawal response recovers with time after the response has reached zero. It is of note that the two measures of recovery used showed that for duration of response, the reflex had not recovered completely by the 20 min post training test, whereas, the magnitude of the post training response had recovered to its original pretraining level by 8 min post training.

## EXPERIMENT 2

Long term habituation of this response from one training session was further investigated by comparing performance of trained snails post-training with that of non-trained snails. The measure used was trials to habituation.

### Method

Subjects. One hundred and eighty mature snails were sampled from a population of 250 snails captured from the hills of Jennycliff Bay, South Devon.

Procedure. Laboratory housing, criterion for participation, test apparatus and habituation stimulus were as reported in Experiment 1. The Experiment was conducted throughout the days of April 18, 19, 20, 1985. Temperature in the laboratory varied from 18 to 23 C.

Fully extended snails were subjected to repeated presentations of the tactile stimulus with an ISI of 30 secs until the criterion for habituation was achieved. Each snail was then numbered for identification with black paint and replaced in the home vivarium. The snails remained in the home vivarium until they were retested for retention. Thirty snails were given an habituation test at each of the following retest intervals, 6, 12, 18, 24 , 36, or 48 hours post training. Of the 30 snails at each retest interval, 15 snails had received one training session to habituation and the remaining 15 snails had received no training prior to the test but were handled. The number of trials required to reach criterion in retest were compared for trained and non trained snails. Evidence of retention was taken as savings in trials to habituation in the trained groups. Trained and non trained snails were all housed together in the same vivarium. Procedure for retest consisted of an assistant locating trained or non trained snails and masking their identification numbers with insulating tape to ensure that the experimenter was unaware whether a snail had been previously trained or not. Habituation procedure and criterion were identical to the initial training phase.

Snails that refused to emerge from their shells in the apparatus orientation period or when due for testing were removed from the experiment and replaced by spare pre-trained snails that fulfilled the retest interval criterion of a particular test. Twelve such substitutions were necessary.

## RESULTS

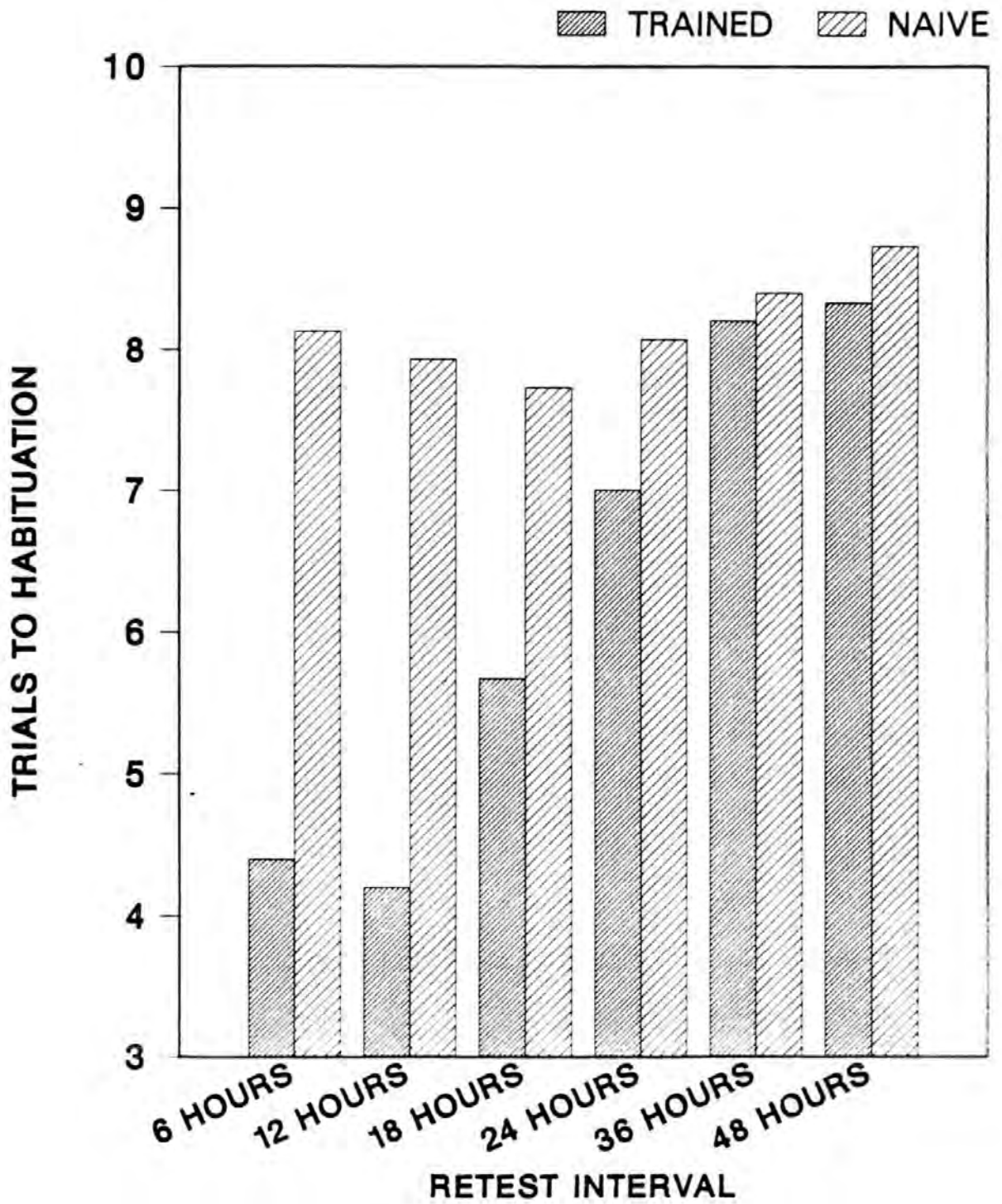
A 2-way ANOVA was conducted with two between subject factors of post training interval and condition (trained/non-trained). This revealed significant main effects of post-training interval ( $F(5/168) = 10.298$ ,  $p < .001$ ) and condition ( $F(1/168) = 48.951$ ,  $p < .001$ ) with a significant post training interval x condition interaction ( $F(5/168) = 5.890$ ,  $p < .05$ ).

Analysis of simple main effects showed that there was no significant difference across post-training intervals for non trained snails ( $F(5/168) = 2.95$ ,  $p > .05$ ), but there was a difference between trained snails at different retest intervals ( $F(5/168) = 77.92$ ,  $p < .001$ ). Savings in trials to habituation decreased as post-training test increased. Trained snails required significantly fewer trials to habituation than non trained snails at the following retest intervals, 6 hrs ( $F(5/168) = 32.58$ ,  $p < .001$ ), 12 hrs ( $F(5/168) = 32.58$ ,  $p < .001$ ) and 18 hrs ( $F(5/168) = 9.94$ ,  $p < .001$ ). There was also a small difference in the 24 hr retest ( $F(5/168) = 2.68$ ,  $p < .05$ ). There was no difference between trained and non trained at 36 or 48 hr retest ( $F(5/168) < 1$ ) (see Figure 4.3).

A further 2 way-ANOVA, with a between subjects factor of post training interval and a within subject factor of session (training/ retest) was conducted on the training and retest data of the trained snails. Trials to habituation in training were compared with the number required in retest, retention at a particular post training interval being

**FIGURE 4.3**

**COMPARISON OF PERFORMANCE OF TRAINED SNAILS WITH NAIVE SNAILS AT SIX RETESTS**



evidenced by savings in retest compared with training. This analysis showed a significant effect of post training interval ( $F(5/84) = 4.580, p < .005$ ) and training session ( $F(1/84) = 175.098, p < .001$ ) with a significant post training interval x training session interaction ( $F(5/84) = 27.972, p < .001$ ).

Analysis of simple main effects revealed large savings in the 6 hr ( $F(5/84) = 118.265, p < .001$ ), 12 hr ( $F(5/84) = 131.95, p < .001$ ), and 18 hr ( $F(5/84) = 57.58, p < .001$ ) retest conditions. Slight savings at retest were evident at 24 hours ( $F(5/84) = 7.117, p < .001$ ). No retention was evidenced at either 36 or 48 hrs ( $F(5/84) < 1$ ). There was no difference in the number of trials to habituation in the initial training sessions ( $F(1/84) = 2.579, p > .5$ ). (see Figure 4.4)

## DISCUSSION

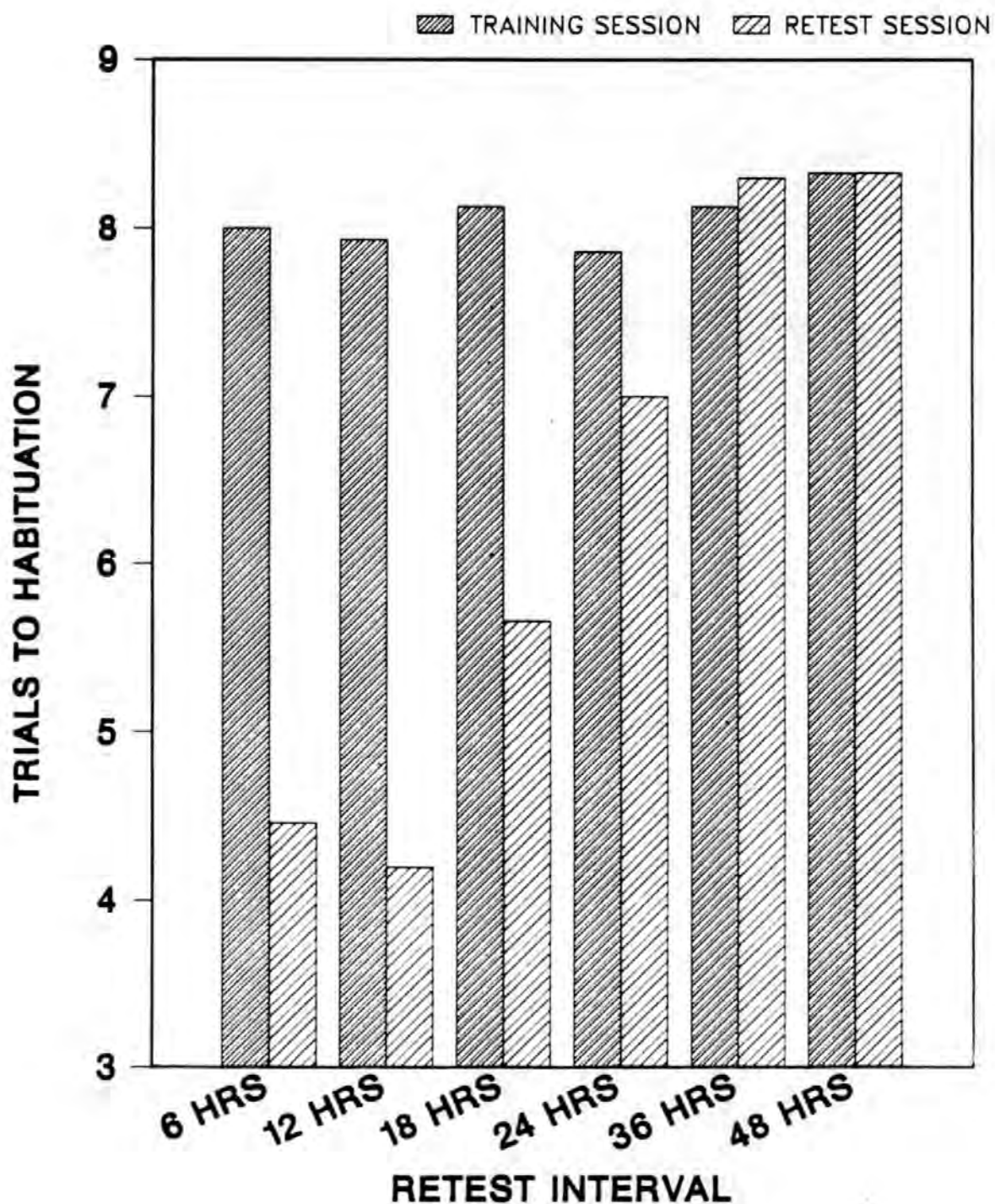
There was evidence of retention in terms of savings on trials to habituation up to 18 hours post training. The amount of saving varied as a function of the post training interval, with greater savings in the shorter post training intervals. At 24 hrs post training there were slight savings evident but these were minimal compared to the savings sampled at 18 hrs post training.

## GENERAL DISCUSSION

The dorsal antennae withdrawal reflex of the snail was found to habituate with an average of 9 presentations of a

**FIGURE 4.4**

**TRIALS REQUIRED TO HABITUATE IN TRAINING AND RETEST  
SAMPLED AT SIX RETEST INTERVALS**



tactile stimulus and that this learning could be retained for between 18 and 24 hrs post training when retention was measured in terms of savings in trials to habituation at retest.

Experiment 1 revealed that the response recovers in terms of the initial response magnitude and duration as a function of time.

These results have implications for two major issues in the the habituation literature. First, habituation is classified as one of the simplest forms of learning and, as a consequence, has often been regarded as a temporary state (Hilgard and Marquis, 1940; Rovold, 1959 and Van Egeran, 1970). The results of Experiment 2 suggest this is a false assumption and support the concept of long term habituation as defined in the parametric characteristics of habituation put forward by Groves and Thompson (1970). Second, the results suggest that long term habituation in the snail can be found after relatively few stimulus presentations, and this retention is comparable to long term data of higher organisms and more complex reflexive behaviours. Thus, the suggestion of Humphrey (1933) and Harris (1943) that phylogenetic position is a major determinant of recovery of the response is not supported.

The retention evidenced in Experiments 1 and 2 of this chapter was produced by one training session. There is evidence from vertebrates (e.g. Farel, 1976) that retention is improved with multiple training sessions. Consequently, the effects of multiple training sessions are investigated in the next section.

THE EFFECTS OF MULTIPLE TRAINING  
SESSIONS ON LONG TERM HABITUATION

Groves and Thompson (1970) suggested that in a series of habituations and response recoveries, habituation becomes successively more rapid. This presupposes a long term effect. Such multiple training session effects have been demonstrated in the mobbing response of Chaffinches (Hinde, 1954), the flexion withdrawal reflex of frogs (Farel, 1976) and the cold pressure and galvanic skin response in Humans (Glaser, 1966) and (Kimmel & Goldstein, 1967).

Unfortunately, many of these studies suffer from poor statistical analysis and/or have failed to demonstrate habituation as opposed to motor or sensory fatigue. Furthermore, the literature on long term habituation effects has utilised a variety of measures which has led to confusion as to the relative completeness of recovery and, consequently, the precise effects of multiple training sessions (e.g Kimmel & Goldstein, 1967).

In this section, a series of 3 experiments are reported which investigate the effects of multiple training sessions on long term habituation of the dorsal antennae withdrawal response using a variety of measures.

EXPERIMENT 3

An initial experiment was conducted to investigate the effects of 2, 1, or no training sessions on long term



retention of the habituation in terms of savings in the number of trials required to reach criterion of habituation at retest.

#### METHOD

Subjects. Two hundred and forty snails were sampled from a population of 420 snails collected from Stonehouse, Plymouth, Devon.

Procedure. The snails were randomly allocated to one of three training conditions. One group received no training, the second received one training session to habituation, and the third received two such training sessions interspaced by 12 hrs. Eighty snails were tested in each training condition. Of these 80 snails, 20 were tested for retention at one of the following post training intervals, 12 hrs, 24 hrs, 36 hrs, or 48 hrs. Laboratory housing, test apparatus, apparatus orientation period, habituation technique, and stimulus were as reported in Experiment 1.

Each snail was numbered for identification with black paint. These numbers were masked with tape at retest by an assistant to ensure that the experimenter was unaware of the training history of any particular snail. All presentations of stimuli had an ISI of 30 secs. Snails that did not emerge on any of the apparatus orientation periods, either in training or retest, were replaced from a population of reserve snails of an equivalent training history. Twenty one such substitutions were necessary.

Snails in the no training group were removed from the

vivarium and placed on the test apparatus twice, interspaced by 12 hrs, to ensure that these snails received the same levels of handling as snails in the training conditions. Snails in the one training condition were removed from the vivarium and placed on the test board where they remained unmolested, they were then replaced in the vivarium for 12 hrs. These snails then were removed again and given one training session to habituation criterion. Snails in the two training session group were removed from the vivarium, tested to habituation, replaced for 12 hrs, and then removed again and given a second training session to habituation.

All post training intervals were timed from the termination of the last habituation session. In no-training sessions, the snail remained on the board for 5 minutes and the post training interval was taken from the completion of this period.

The number of trials taken to reach criterion of habituation were recorded for each snail in each training session. The Experiment was conducted during the days of April 23, 24, 26, 28, 1985. Temperature in the laboratory varied between 14 and 20 C.

## RESULTS

The results were analysed in a 2-way ANOVA with between subject factors of training (1, 2, or no sessions), and retest (12, 24, 36, or 48 hrs). This revealed a significant effect of training ( $F(2/228) = 56.227, p < .001$ ), but no significant effect for retest ( $F(3/228) < 1$ ) or training x

retest interval interaction ( $F(3/218) < 1$ ).

The results of this experiment are reported in Figure 4.5 and show that in the 12 hr retest, greatest number of trials were required to reach criterion in the no training group and least were necessary in the two training group. At 24 hrs post training, retention was only evidenced by snails that had received two training sessions, as was the case at 36 and 48 hrs post training.

In order to investigate the effects of multiple training sessions on the number of trials required to reach criterion of habituation in successive training sessions and at retest, data from the two training session snails were analysed in a separate 2-way ANOVA with a within subject factor of training session and a between factor of retest. This was conducted to show savings in trials to habituation in successive sessions. This revealed a significant effect of training session ( $F(1/76) = 175.807, p < .001$ ), and no effect of retest interval ( $F(3/76) < 1$ ) with retention evident at all retest intervals. There was no significant training session x retest interval interaction ( $F(6/76) < 1$ ). A saving in trials to habituation was evident in each successive session.

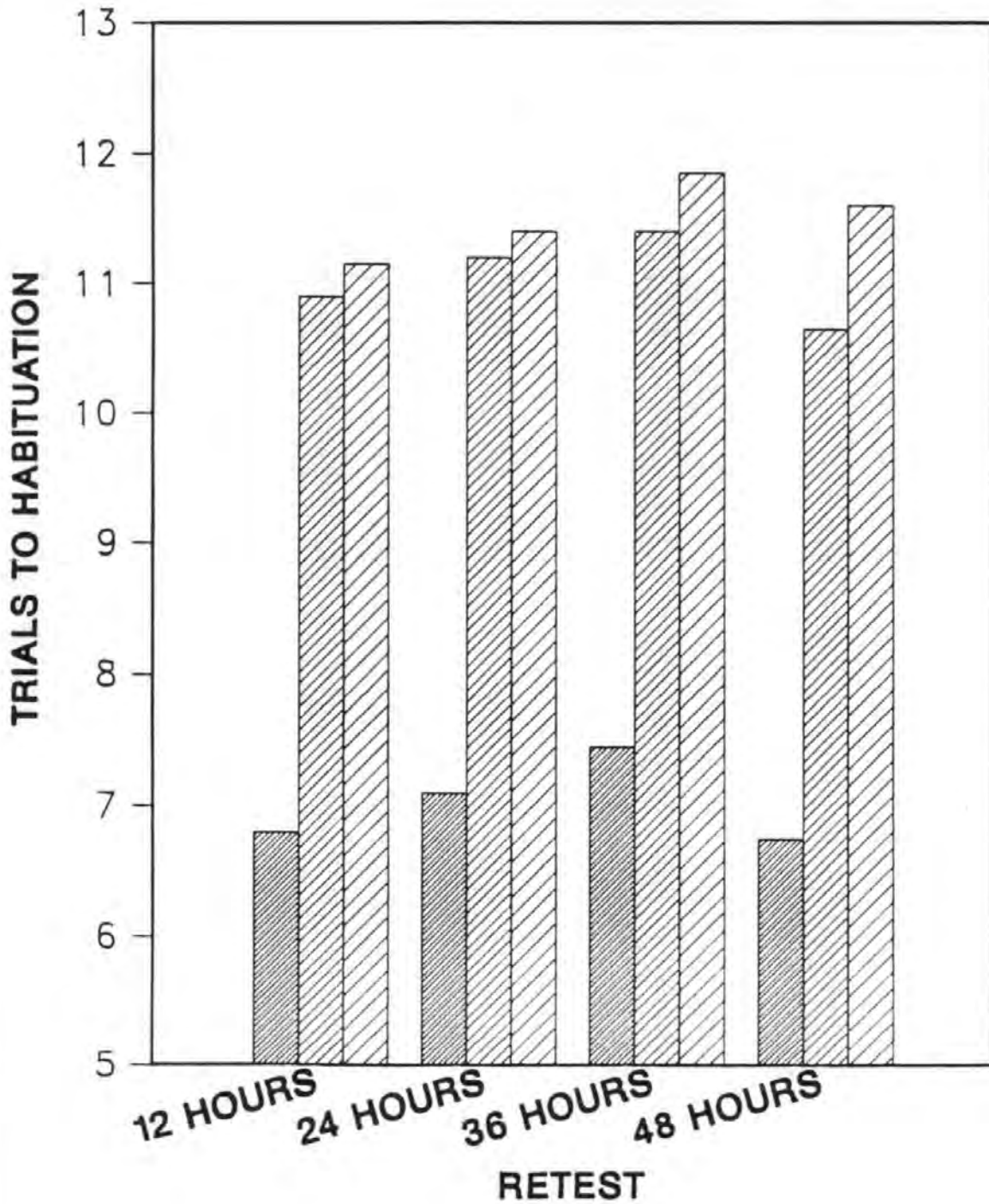
## DISCUSSION

The results of Experiment 3 show clear evidence of greater savings for snails that had received two training sessions compared to snails that have received one training session which, in turn, showed more savings than snails with

FIGURE 4.5

LONG TERM HABITUATION AT THREE RETESTS

▨ TWO TRAINING SESSIONS    ▩ NO TRAINING  
▧ ONE TRAINING SESSION



no previous training.

#### EXPERIMENT 4

Trials to a criterion of habituation as a training procedure has one major short-coming. The results on retest may be confounded by differential levels of stimulus exposure in training. If snails take more trials to habituate, their objective experience of the stimulus is greater than snails that required fewer trials.

Furthermore, although trials to habituation seems to be a more sensitive measure of retention, it does not give information concerning levels of response which is provided by Experiment 1.

To overcome these problems, Experiment 3 reports the effects of two, fixed number of trials, training sessions on retention of habituation. Individual responses elicited by each stimulus were recorded in the retests to assess long term habituation in terms of level of responses shown at various post training intervals.

#### METHOD

Subjects. Thirty snails were tested from a population of laboratory bred snails housed in a glass vivarium (1 x 1 m).

Procedure. The stimulus and procedure was the same as that used in Experiment 3. The experiment was conducted during April 29, 30, and May 1, 1985. All snails received

two training sessions interspaced by 12 hrs which were spent in the home vivarium. Each training session consisted of a numbered snail being removed from the home vivarium and placed on a wooden test board. This apparatus orientation period was as reported in Experiment 1. Snails that were fully extended received 10 stimulus presentations. Upon completion of the final response they were replaced in the home vivarium until the next session in which the procedure was repeated.

Retesting the snails involved the same procedure as the training sessions. Ten snails were sampled for retention of habituation at each of the following post training intervals 12 hrs, 24 hrs, or 48 hrs. The 10 responses elicited by snails in each retest were recorded in terms of their duration (see Chapter 3).

The development and retention from each session was measured in terms of individual response magnitudes and durations.

Snails that did not fulfill the participation criterion were removed from the study and replaced with snails at an equivalent point in training. Six such substitutions were necessary.

## RESULTS

Duration of response data for the three retests were analysed in a 2 way ANOVA with a between subjects factor of retest interval and a within subject factor of trial. The main effects revealed were a significant effect of retest

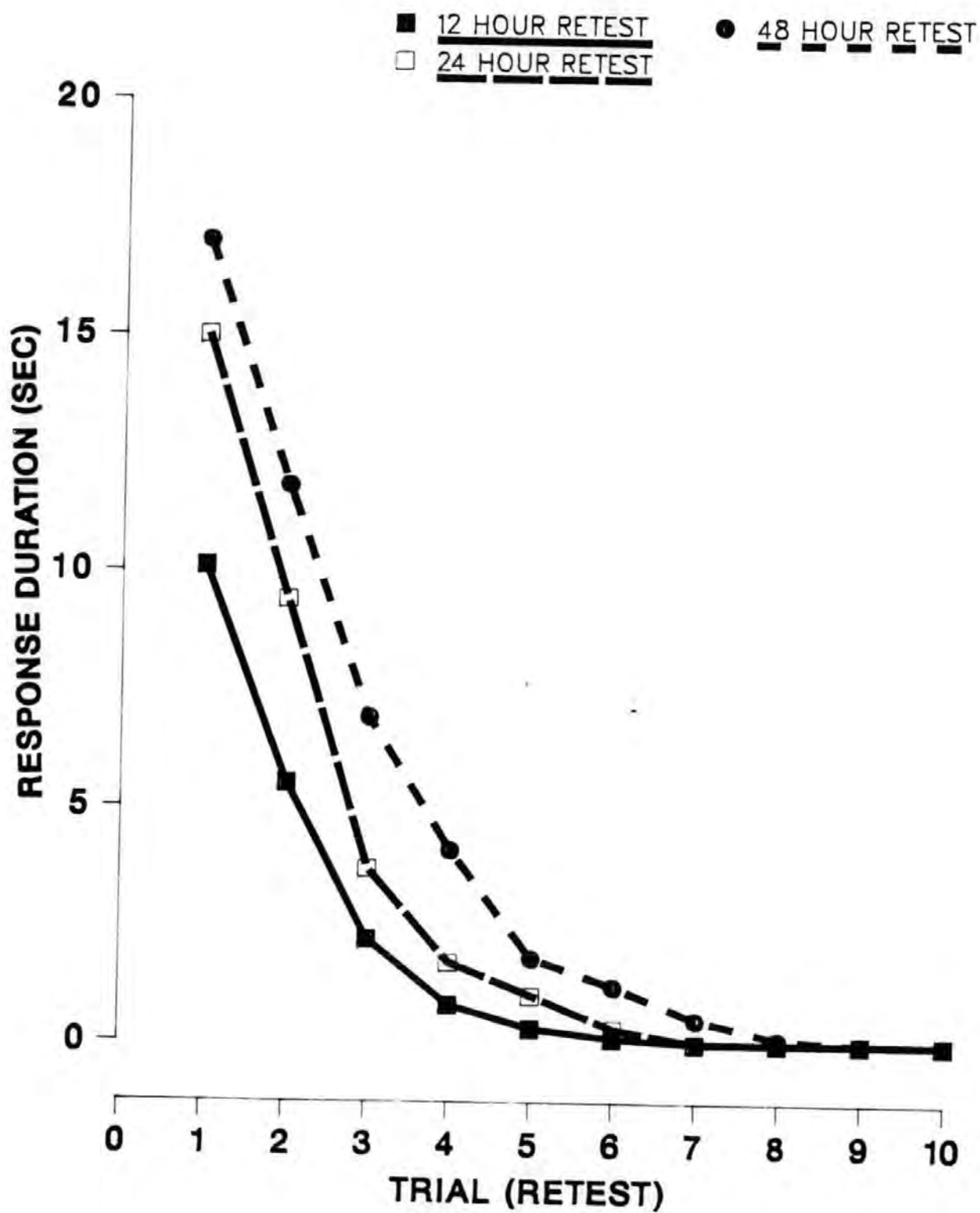
interval ( $F(2/27) = 14.977, p < .001$ ) and of trial ( $F(1/27) = 253.522, p < .001$ ) with a significant retest interval  $\times$  trial interaction ( $F(2/27) = 7.268, p < .01$ ).

Further analysis revealed a decrement of responses with successive stimulus presentations in the 12 hr ( $F(2/27) = 380.34, p < .001$ ), 24 hr ( $F(2/27) = 875.92, p < .001$ ) and the 48 hour ( $F(2/27) = 1156.05, p < .001$ ) retests. Comparisons between retest conditions at the level of individual responses revealed smallest response durations were elicited in the 12 hour post-training group and the largest were evidenced at 48 hours post training on Trials 1 to 5 ( $F's(2/27) = 104.72, 84.01, 47.89, 24.18, p < .001$  and  $4.69, p < .025$  respectively). With Trial 6 to Trial 10 the responses were close to asymptote. Consequently, although responses were largest in the 48 hr group and smallest at 12 hr retest, the difference failed to reach significance. The results are illustrated in Figure 4.6.

Analysis of the response magnitude data in an identical ANOVA revealed a significant effect of retest interval ( $F(2/27) = 6.382, p < .001$ ) and trial ( $F(1/27) = 203.673, p < .001$ ) with no retest interval  $\times$  response interaction ( $F(2/27) = 1.891, p > .05$ ). Results on this measure again showed that retention decreased as post training interval increased. The initial response magnitudes did not significantly vary between retest conditions. However, as with response duration data, magnitude of response did vary in Trials 2 to 7. Greatest response magnitudes were evidenced in the 48 hour test, smallest response magnitudes were shown in snails tested 12 hours post training. These

FIGURE 4.6

LONG TERM HABITUATION AT THREE RETESTS





effects are illustrated in Figure 4.7.

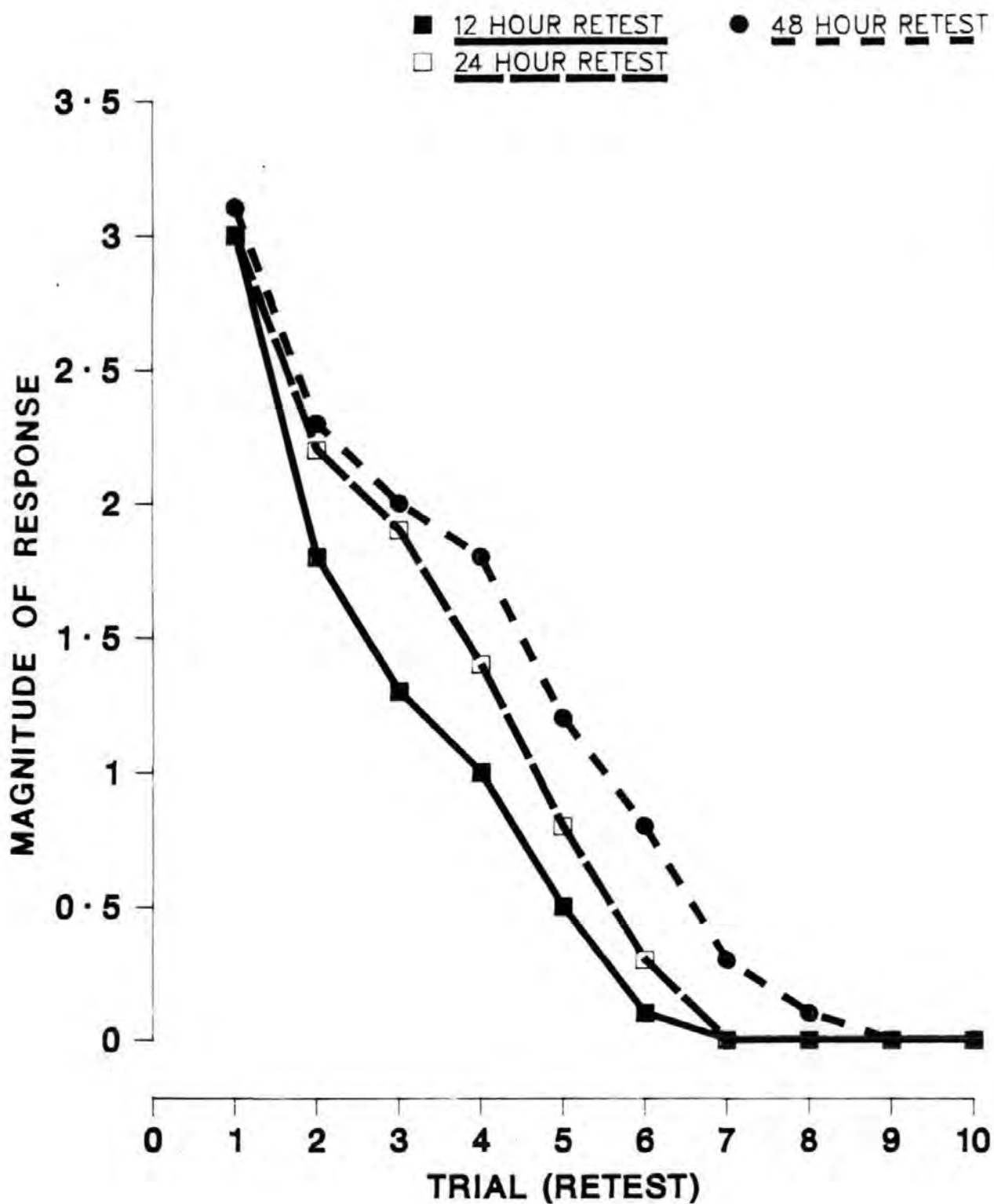
The duration of response to the first stimulus presentation in each of the training sessions and the retest were also analysed. A 2-way ANOVA with a within subjects factor of session (training 1, training 2, and retest) and a between subjects factor of retest interval showed a significant main effect of session ( $F(1/27) = 208.125$ ,  $p < .001$ ). There was no effect of retest interval ( $F(2/27) < 1$ ), but there was a significant session x retest interval interaction ( $F(2/27) = 9.445$ ,  $p < .001$ ).

Further analysis revealed a successive decrement in first response durations across repeated sessions in all three training conditions ( $F's(2/27) = 252.49$ ,  $111.71$ , and  $89.80$ ,  $p < .001$ ). There was no difference between groups in the initial response duration of training Session 1 ( $F(2/27) < 1$ ), or Session 2 ( $F(2/27) = 1.27$ ,  $p > .05$ ). However, there was a large difference between groups at retest ( $F(2/27) = 72.52$ ,  $p < .001$ ). Largest initial responses were elicited in the 48 hr group and smallest in the 12 hr group. Thus, using this measure, retention of learning was evident at all retests with least retention at 48 hrs and greatest retention at 12 hrs post training.

Analysis of the magnitudes of the same responses in an identical ANOVA showed a significant effect of session ( $F(1/27) = 39.470$ ,  $p < .001$ ), but no effect of retest interval ( $F(2/27) = 1.035$ ,  $p > .5$ ) or session x retest interval interaction ( $F(2/27) < 1$ ).

**FIGURE 4.7**

RESPONSE MAGNITUDES AT THREE RETESTS  
FOR LONG TERM HABITUATION



## DISCUSSION

Snails sampled for retention at 12 hrs post-training responded with smaller response durations and magnitudes. Although there was evidence of long term habituation from two, fixed number of trials, training sessions at 48 hrs post-training, the largest responses were evidenced at this time. Therefore, as expected, retention was seen to be to vary with elapsed, post-training, retest time.

## EXPERIMENT 5

The experiments reported in this section all explore the parameters of long term habituation from 2 training sessions up to two days post-training. Experiment 5 was conducted to study the effects of more than two training sessions on long term retention of habituation at 3 weeks post-training.

### Method

Subjects. Forty mature snails were sampled from a population of 100 snails collected from Bovisand Bay South Devon (U.K.). These snails were housed in a glass vivarium (1 x 1 m) containing a grass sod, under loose moist vegetation and two petri dishes of water. The diet of the snails was supplemented by a lettuce that was placed in the vivarium weekly.

Procedure. Ten snails were randomly allocated to each

of 4 training conditions: either 1, 2, 4, or 6 training sessions. Each training session consisted of 10 presentations of the tactile stimulus with an ISI of 30 secs and an intersession interval of 12 hrs. Snails in all conditions were sampled for retention three weeks after the last training session. Training sessions and the three week retest were preceded by an apparatus orientation period of 1 min. Failure to emerge during this period terminated that animals participation in the study. All snails were numbered for identification and reserve animals were trained such that they could replace snails that did not fulfill the criterion for continued participation. Eight such substitutions were made. The habituating stimulus and training procedure were as reported in Experiment 1. In all intersession intervals and the interval between last training session and retest snails were replaced in the home vivarium. Performance at retest was compared.

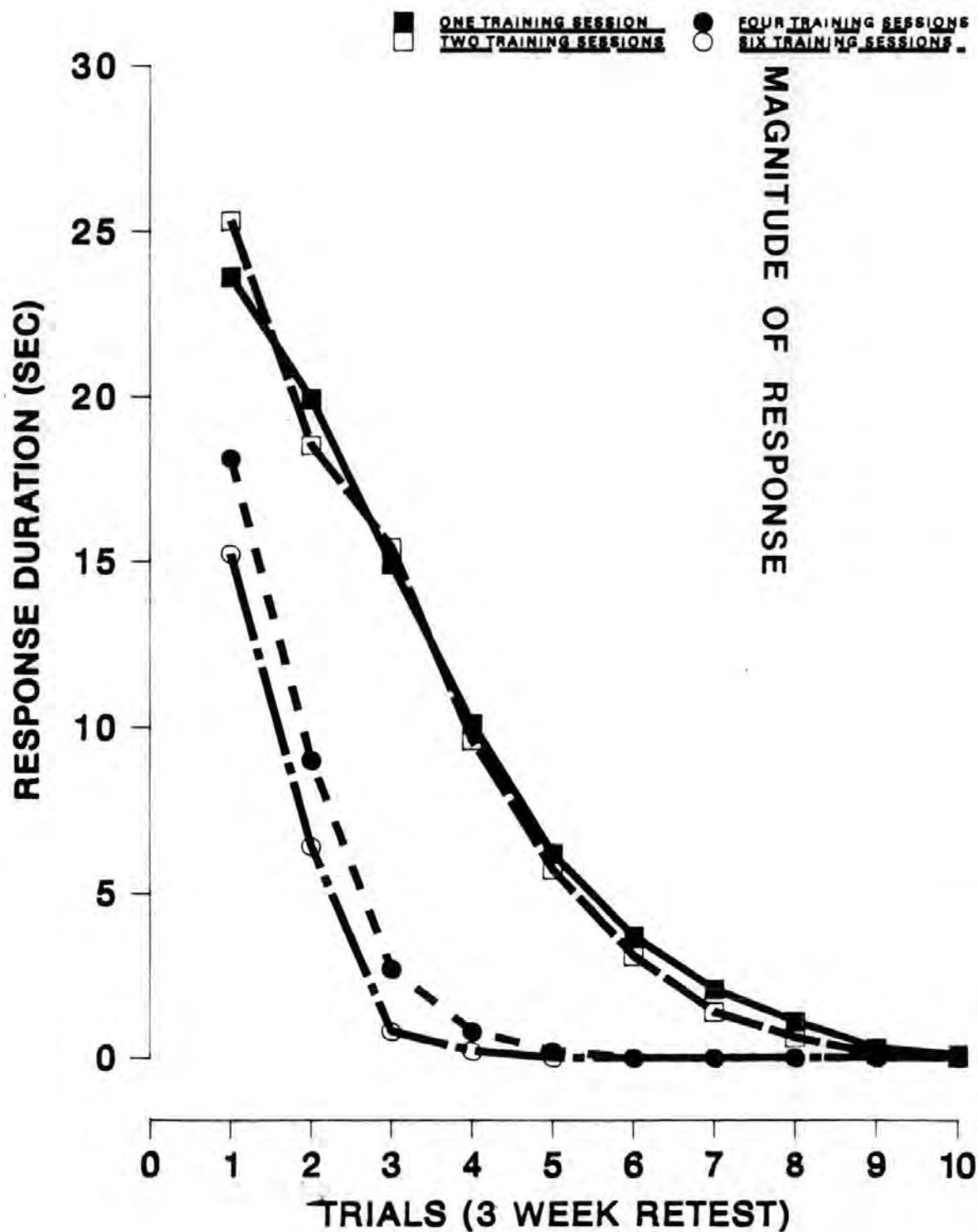
## RESULTS

The results of Experiment 5 are depicted in Figure 4.8. Duration of the 10 retest responses were analysed in a 2-way ANOVA with a between subjects factor of amount of training, and a within subject factor of trial (1 to 10). This revealed significant effects of amount of training ( $F(3/36) = 95.447, p < .001$ ) and trials ( $F(1/36) = 841.203, p < .001$ ) with a significant number of training x trial interaction ( $F(3/36) = 37.225, p < .001$ ).

Further analysis showed that at all 10 stimulus

FIGURE 4.8

RETENTION AT THREE WEEKS POST-TRAINING FROM ONE, TWO, FOUR OR SIX TRAINING SESSIONS



presentations there was a difference in the response duration dependent on the amount of prior training until stimulus 10 when all snails were at zero response duration (see Figure 4.8). Snails that received either 1 or 2 training sessions showed largest response durations, and smallest response durations were found with snails that received 6 training sessions.

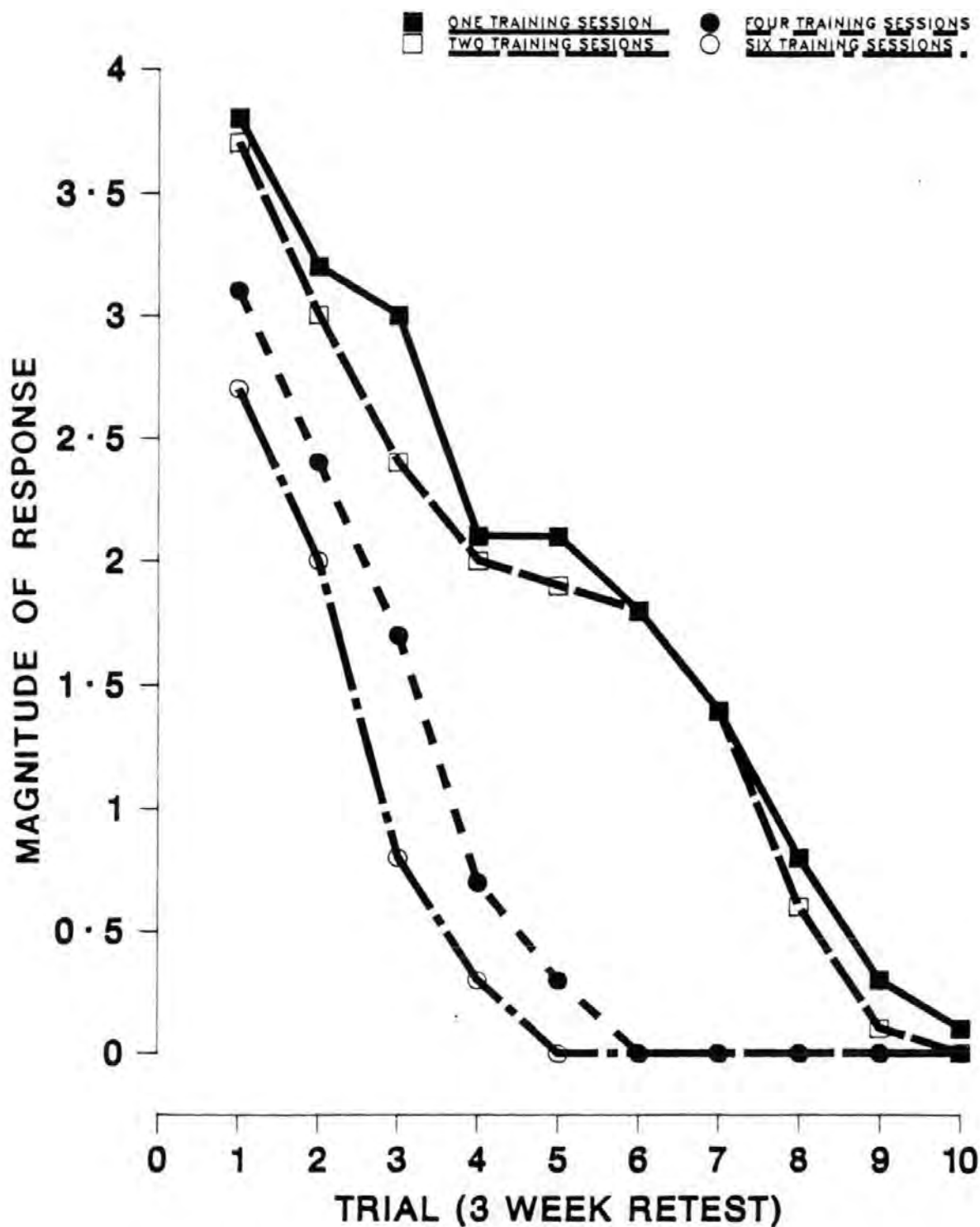
The magnitude of retest response data were similarly analysed revealing similar results to the above measure with significant effects of training ( $F(3/39) = 102.395, p < .001$ ) and trial ( $F(1/35) = 315.526, p < .001$ ) and a significant interaction of number of training x trial ( $F(3/35) = 10.076, p < .001$ ). The results on this measure support the conclusions of the further analysis made on the duration of response data. Results for this measure are reported in Figure 4.9. Largest response magnitudes were evidenced from snails that had received 1 training session, smallest response magnitudes shown by snails that received 6.

## DISCUSSION

Experiment 5 produced evidence for retention of habituation up to 3 weeks post training in snails that received 4 or 6 training sessions. Levels of responses at retest varied as a function of number of training session. The more training sessions that were given, the less recovery of response. It is interesting to note that the levels of response in retest for the 1 and 2 session groups are the same as those evidenced in initial training in

FIGURE 4.9

RETENTION OF HABITUATION AT THREE WEEKS POST-TRAINING FROM ONE, TWO, FOUR OR SIX TRAINING SESSIONS



Experiment 4. Thus it could be concluded that snails in these groups had shown complete recovery i.e. showed no retention of habituation.

#### EXPERIMENT 6

The above experiments all serve to demonstrate long term habituation of the dorsal antennae withdrawal response in Helix aspersa and show both multiple training effects on retention and that the retention is comparable to retention evidenced in vertebrates. In order to extend these findings, retention in terms of months was investigated after multiple training sessions.

#### METHOD

Subjects. Two hundred and forty snails were studied, collected from a Plymouth garden and housed in a glass vivarium 80 x 35 cm in the laboratory. Each animal was assigned a number for identification which was painted on the shell and coated with varnish. Food was available throughout the experiment. The snails were allowed to feed naturally on loose vegetation placed in the vivarium every 48 hrs.

Procedure. The was conducted throughout the days of October 31, November 1 to 26, 1984. January 2 to 26, and May 2 to 26, 1985. Temperature in the laboratory varied from 15 to 23 C. At the commencement of each training session, the subject was removed from the home vivarium and placed on a



test board measuring 30 x 25 cm of wood (pine) construction. Snails were randomly allocated to sixteen groups of fifteen animals each. Four groups received 1 training session, of these, one group was retested at 1 week, 3 weeks, 2 months, and 6 months post training. Similarly, 4 groups received 4, 6, and 10 training sessions with one of each of the four groups sampled for retention at each of the retest intervals. Training commenced one minute after the snail was placed on the board if the snail was fully extended from its shell. Any snail which appeared dormant at the commencement or during training or retest was discarded from the data analysis and replaced by reserve animals collected from the same site at the same time and housed with the test population. Forty six substitutions were necessary. Training was with the tactile stimulus used in Experiment 1, Chapter 3. Stimulus presentation and procedure were as reported in the initial experiment of this chapter. The number of trials each snail required to reach criterion, exclusive of the three non responses, was recorded for each training session. Upon completion of each training session, the snail was replaced in the home vivarium for the 12 hr inter-training interval. Retest sessions used the same procedure, and were conducted by a second experimenter naive as to the training regime a particular snail had undergone. Trials to habituation at retests were compared for groups that had received different numbers of training sessions.

## RESULTS

The retest scores from all groups were analysed in a 2-way ANOVA with between subject factors of training and retest interval. This yielded a significant effect of training condition ( $F(3/239) = 335.149, p < .001$ ) and retest interval ( $F(9/239) = 95.005, p < .001$ ) with a significant training  $\times$  retest interval interaction ( $F(9/239) = 29.586, p < .001$ ). This is illustrated in Figure 4.10.

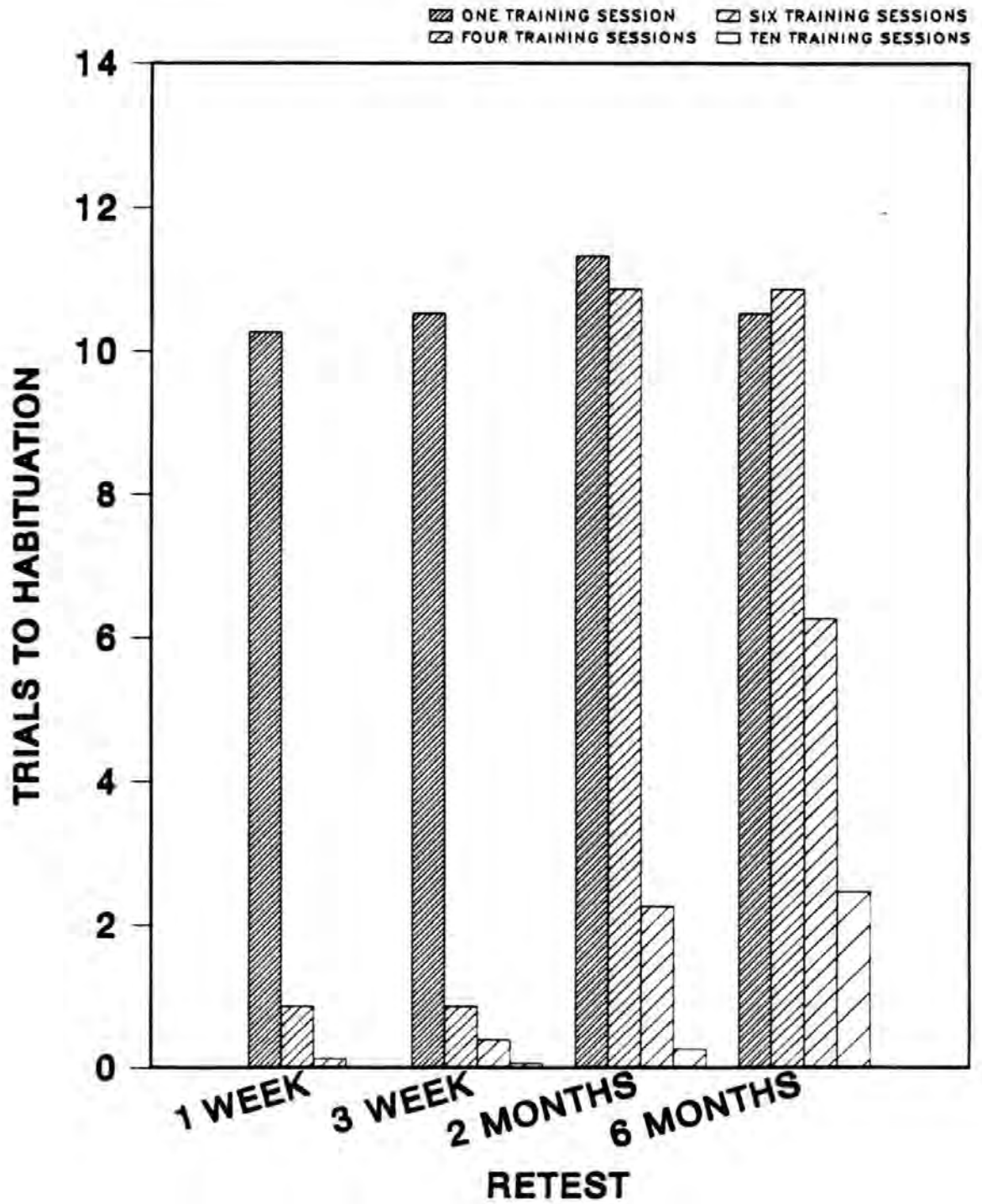
Analysis of simple main effects revealed a significant difference between training conditions in all retests ( $F(9/239) = 542.841, p < .001$ ). Comparison between training conditions in the one week retest showed a significant difference ( $F(9/239) = 318.62, p < .001$ ) in trials to habituation at this retest interval. Snails in the 1 training session condition required an equivalent number of responses at retest ( $M = 10.27$ ) as they did in training ( $M = 10.63$ ). Profound retention was evidenced by snails that had received 4 sessions ( $M = 0.87$ ), 6 training sessions ( $M = 0.13$ ) and 10 sessions ( $M = 0.00$ ).

In the 3 week retest, there was also a difference between training conditions ( $F(9/239) = 327.54, p < .001$ ). Snails that had been trained to criterion once, showed no retention in this retest ( $M = 10.53$ ). Again, there was a large saving in trials to habituation at retest in snails that received 4 sessions ( $M = 0.87$ ), 6 sessions ( $M = 0.40$ ) and 10 sessions ( $M = 0.07$ ).

By 2 months post training there was no retention evidenced by snails that received 1 session ( $M = 11.33$ ) or 4

**FIGURE 4.10**

**THE EFFECTS OF DIFFERENT NUMBER OF TRAINING SESSIONS ON RETENTION AT FOUR RETESTS**



training sessions ( $M = 10.86$ ). Retention was evident in snails from the 6 ( $M = 2.27$ ), and 10 session conditions ( $M = 0.27$ ).

The final retest interval sampled was 6 months post training. Here, no retention was evident in snails that received 1 or 4 training sessions ( $M$ 's = 10.53 and 10.87). Some retention was shown by snails in the 6 session condition ( $M = 6.27$ ), and, interestingly, the snails that had received 10 training sessions to criterion of habituation still retained the training ( $M = 2.47$ ).

## DISCUSSION

It can be seen that there is overwhelming evidence for long term habituation. Snails that received one training session showed no retention of the learning at 1 week, 3 weeks, 2 months, or six months. Snails that received four training sessions, demonstrated retention at 1 week and 3 weeks with no evidence of retention at 2 months or 6 months post training. Snails that received six and ten training sessions showed retention at 1 week, 3 weeks 2 months, and six months.

## GENERAL DISCUSSION

The antennae withdrawal reflex in the terrestrial snail Helix aspersa shows long term habituation and this retention is greater after multiple training sessions. Such retention, as evidenced in these six experiments, firmly establishes

habituation of the dorsal antennae withdrawal response as a 'true' form of learning and equates the parameters of this learning with those found in vertebrate habituation studies (See Chapter 2).

From a variety of measures of habituation, the above research shows that habituation can be retained between 18 and 24 hrs from one session of training to habituation or from a fixed number of 10 stimulus exposures. Retention from two training sessions is evident up to two days post-training and with four or six training sessions there is retention three weeks post-training. With six or ten training sessions, retention is evident six months post-training.

Interestingly, the first response of retest indicates response recovery in minutes in the snail. However, further investigation utilising other measures revealed that some retention did occur long after this. This may be a consequence of differential processing in short and long term habituation. The initial recovery measured in Experiment 1 may reflect the initial waning of a short term phenomenon, and this short term process may not reflect the long term processing of the same learning. This interpretation supports a differential process view of habituation (see Chapter 2). However, it may just be that magnitude of first response in retest is not as sensitive a measure of habituation as are measures of development of habituation during retest.

Having established habituation of the dorsal antennae withdrawal response in the snail and explored its long term

effects, other parametric characteristics of habituation such as ISI effects and other forms of massed and spaced training that have been reported in vertebrates are examined in the snail in subsequent chapters.

## CHAPTER 5

### THE EFFECTS OF INTERSTIMULUS INTERVAL ON SHORT AND LONG TERM HABITUATION

As outlined in Chapter 2, it has been suggested that short and long term habituation are mediated by different mechanisms. This conclusion is based, in part, upon differences in the parametric characteristics of short and long term habituation and has been supported by differential effects of PSIs on short and long term habituation.

Much of the work on these differences has centred upon the effects of various manipulations of stimulus frequency. The relationship between ISI and habituation has generated a prolific literature (see Stephenson, 1982). In animals and humans, shorter ISIs have consistently been reported to produce faster habituation than long ISIs. Although the direct relationship between ISI and short term habituation appears incontrovertible, the effects of ISI on long term habituation or delayed habituation remains equivocal.

Pieron (1913) and Goodman and Weinberger (1973) demonstrated that the relationship between trials to habituation and ISIs in short term habituation did not hold true for long term habituation. Askew (1970) reported that in head shake response in the rat, short ISIs were more effective in the production of short term habituation. However the larger ISIs produced no recovery when tested 30 mins post training. Similarly, Ray (1979) found that short ISIs required fewer trials to habituation than did long

ISIs, and reports that there was no difference between the two conditions he used in a long term test. Szlep (1964), however, reported faster habituation with short ISIs in both short and long term habituation in the spider Araneus. The somewhat disparate and confused nature of long term ISI effects is due, in no small part, to the diversity of experimental designs, methodologies and animals in which these effects have been studied. There is a need for evidence of the effects of ISI on both short and long term habituation in the same animal response system, with a uniformity of measures of habituation in both.

A series of experiments investigating these effects of ISI on short and long term habituation of the dorsal antennae withdrawal response in *Helix* are reported in this chapter.

## 5:1 THE EFFECTS OF ISI ON SHORT TERM HABITUATION

### EXPERIMENT 1

An initial study investigated the relationship between ISIs and the number of trials required to establish short term habituation of the dorsal antennae withdrawal response.

Speed of habituation (i.e. the number of trials required to reach criterion of habituation) is probably the simplest measure of habituation, and it has been widely used in the study of ISI effects on habituation in preparations ranging from worms (Gardner, 1964) to humans (Ray, 1979). Research suggests that short ISIs require fewer trials to



reach criterion of habituation than long ISIs. This was tested in the snail.

### Method

Subjects. Two hundred and eighty mature snails collected from the Bovisand Bay area of Plymouth (U.K.) were laboratory housed in two glass vivaria (50 x 50 cm). Each vivarium contained a grass turf and loose vegetation. The snails' diet was supplemented by lettuce leaves placed in each vivarium every 48 hours. The Experiment occurred between 2400 and 1030 hrs on the mornings of March 9, 10, and 11, 1985. Temperature in the laboratory varied from 18 to 23 C.

Procedure. After a one week acclimatisation period, the data collection phase commenced. Two hundred of the collected snails participated in the study. Each snail was in turn removed from its home vivarium and placed on a varnished pine-wood test board (30 x 30 cm) where it remained unmolested for 60 secs. If, after this apparatus orientation period, the snail was fully extended from its shell, it was used in the experiment. Forty snails were randomly assigned to each of five ISI conditions of 10, 20, 30, 60 and 300 secs. ISI was calculated from termination of a response i.e. when the right antennae had recovered its full posture, to the presentation of the next stimulus.

Twenty snails in each condition received repeated presentations of the tactile stimulus, as described in Experiment 1 of Chapter 3, at the given ISI until criterion

of habituation had been reached. The remaining snails received the same ISI treatments but with the water drop stimulus described in Experiment 4, Chapter 3.

## RESULTS

These data were analysed in a 2-way ANOVA with between subject factors of ISI and stimulus type. This revealed a significant effect of ISI ( $F(4/190) = 134.16, p < .001$ ) There was no significant effect of stimulus type ( $F(1/190) = < 1$ ) and no significant ISI x stimulus type interaction ( $F(4/190) < 1$ ). Trials required to reach criterion of habituation increased as ISI increased. This relationship was found to be true for both stimulus types (see Figure 5.1).

## DISCUSSION

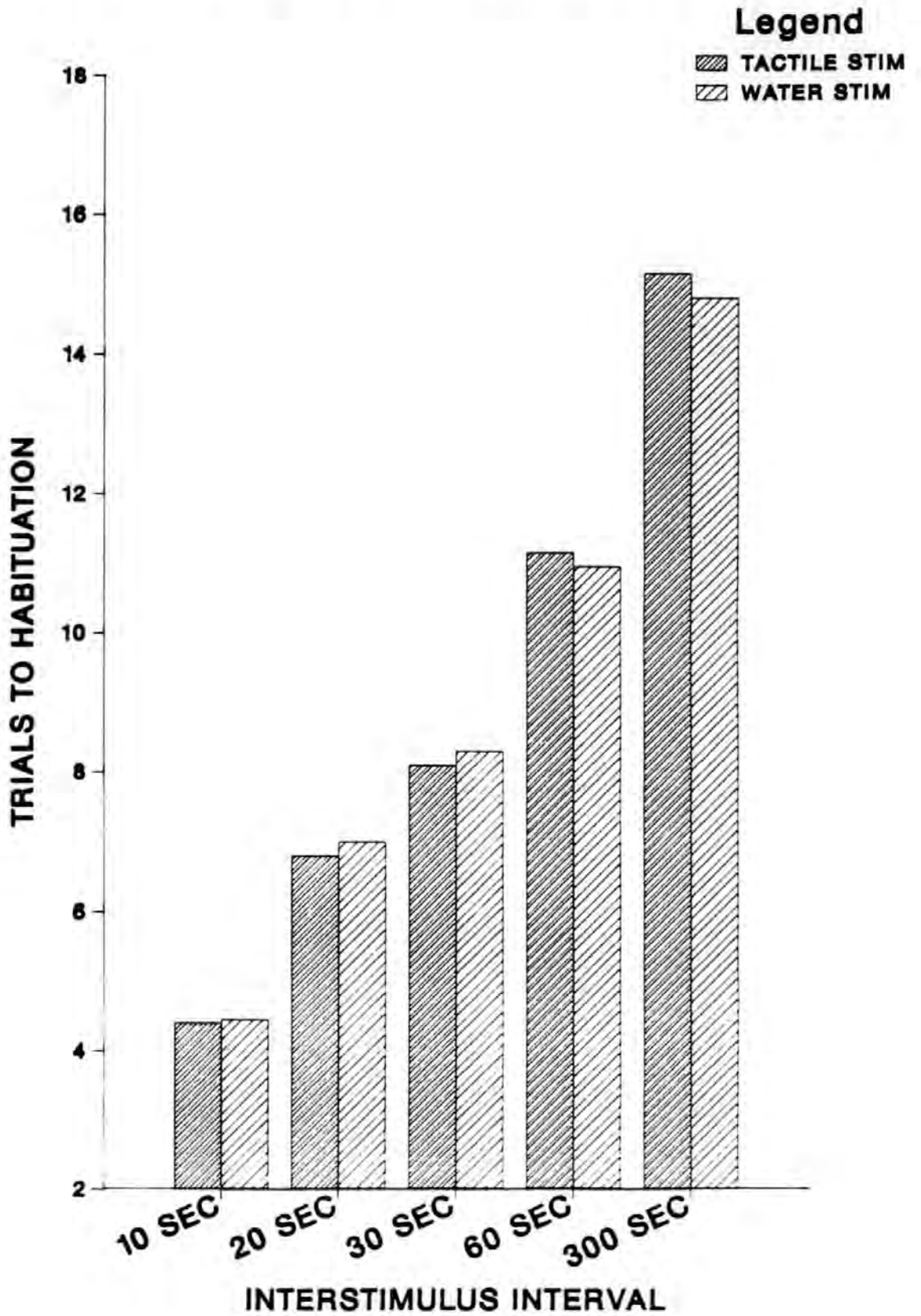
In all ISI conditions, habituation to criterion was found, and there was a difference in trials required to habituate between ISI conditions. The results suggest that the effects of ISI on speed to habituate in short term habituation of the antennae withdrawal response in Helix aspersa are in accord with the rest of the literature cited by Stephenson (1982). That is, short ISI produce faster habituation.

## EXPERIMENT 2

While the above study concurs with the relationship of

FIGURE 5.1

THE EFFECTS OF ISI ON SHORT TERM HABITUATION



ISIs with short term habituation advocated by the literature, only one measure of habituation was applied. Consequently, a second experiment was conducted to investigate the effects of ISI on short term habituation using a different measure of habituation.

#### METHOD

Subjects. Thirty mature snails were collected from a population snails resident in the Tamerton Valley area of Plymouth (U.K.), and housed in an identical way as reported in Experiment 1. All snails were selected for participation in the experiment on the criterion of being fully extended after the apparatus orientation period and throughout the test.

Procedure. The experiment was conducted between 0100 and 0830 Hrs on the mornings of March 26, 28, 29, 1985. Temperature in the laboratory remained constant at 20 C. The snails were randomly allocated to one of three ISI conditions of 10, 30 or 60 secs. Ten snails per condition each received the tactile stimuli described in Experiment 1, Chapter 3. The procedure, and stimulus was identical to that of Experiment 1, except each snail received a fixed number of trials (10). Duration of response scores were recorded for each stimulus presentation, each response was also categorised in terms of magnitude of the response. Response measurement was as reported in Chapter 3. Within the confines of the fixed number of trials design, trials to habituation were also analysed.

## RESULTS

The effects of ISI on acquisition curves for short term habituation are illustrated in Figure 5.2.

Analysis of response duration scores in a 2-way ANOVA with a between subjects factor of ISI and a within subject factor of trials showed a significant effect of ISI conditions ( $F(2/27) = 29.817, p < .001$ ) and trial ( $F(1/27) = 441.913, p < .001$ ) with a significant ISI x trial interaction ( $F(2/27) = 8.872, p < .01$ ).

Analysis of simple main effects revealed that all ISI conditions showed a decrement in response duration with repeated trials ( $F's(2/27) = 1480.18, 1376.73, \text{ and } 3335.64, p < .001$ ) for ISI of 10, 30 and 60 sec. These acquisition curves varied with ISI (see Figure 5.2). At the level of individual trials across ISI conditions there was no significant difference between ISI conditions at Trial 1 ( $F(2/27) < 1$ ). There was, however, a difference on all the other trials except Trial 10.

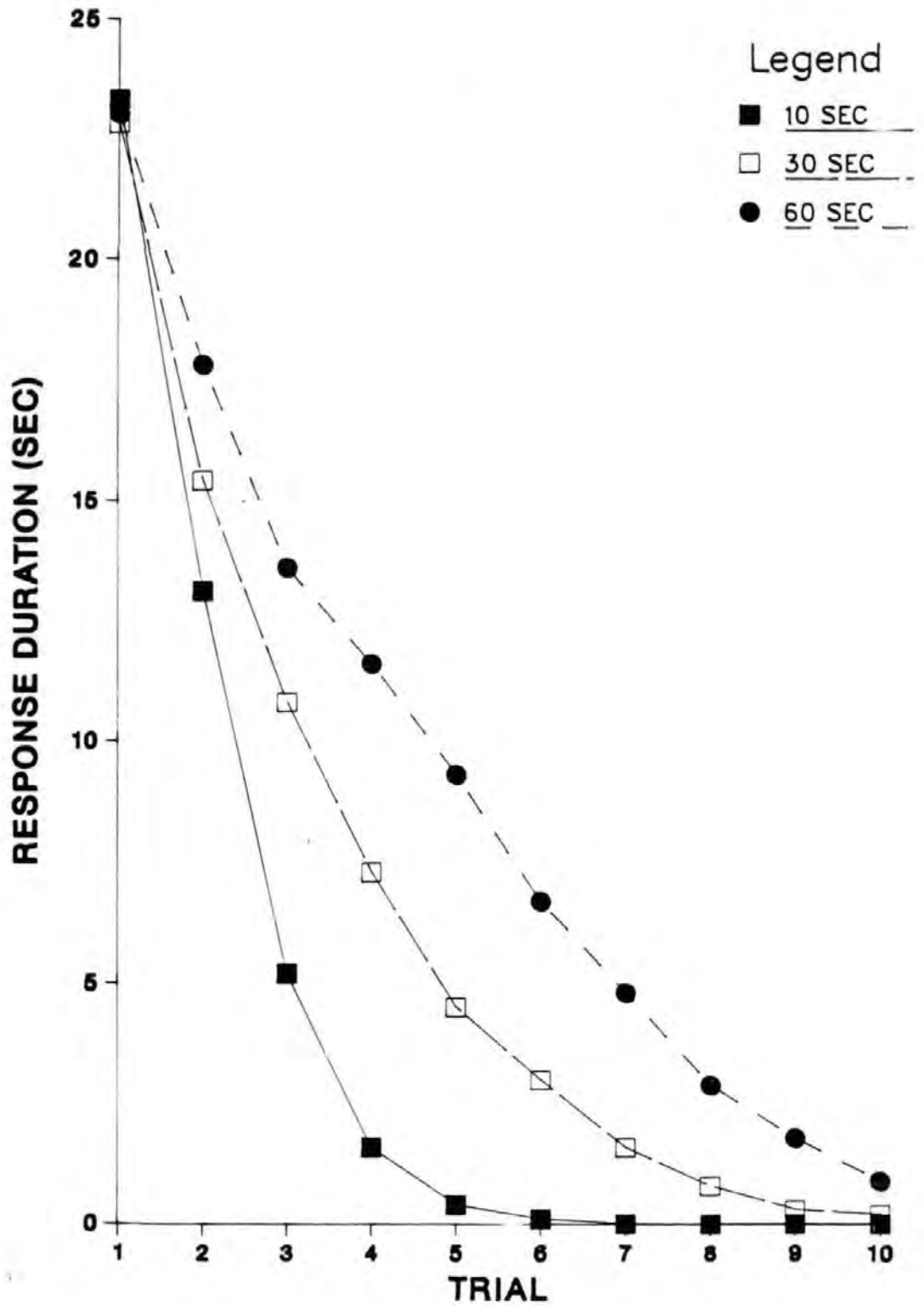
TABLE 5.1.

COMPARISONS OF INDIVIDUAL RESPONSES BETWEEN  
ISI CONDITIONS

TRIAL	D/F	F VALUE	SIGNIFICANCE
1	2/27	0.37	$p > .5$
2	"	32.83	$p < .001$
3	"	108.75	$p < .001$
4	"	149.59	$p < .001$
5	"	117.97	$p < .001$
6	"	65.06	$p < .001$
7	"	35.53	$p < .001$
8	"	13.34	$p < .001$
9	"	5.53	$p < .01$
10	"	1.33	$p > .5$

FIGURE 5.2

# ISI AND RESPONSE DECREMENT



Smallest response durations at all levels of trial were evidenced by snails in the shortest ISI condition (30 secs), and largest responses were found in the longest ISI condition (60 secs).

Category of response magnitude scores were also analysed in a 2-way ANOVA and showed a significant effect of ISI ( $F(2/27) = 175.429, p < .01$ ) and trial ( $F(1/27) = 251.573, p < .001$ ) with a significant ISI x trial interaction ( $F(2/27) = 10.669, p < .001$ ).

Analysis of simple main effects for this measure revealed the same pattern of results as that for duration. There was a decrement in response magnitude in all ISI conditions. Only the first response showed no variance between ISI conditions ( $F(2/27) < 1$ ). At all other trials there was a significant difference (see Table 2) between the three ISI conditions. Again, short ISIs showed smaller response magnitudes, at all these levels. These results are illustrated in Figure 5.3. & Table 5.2.

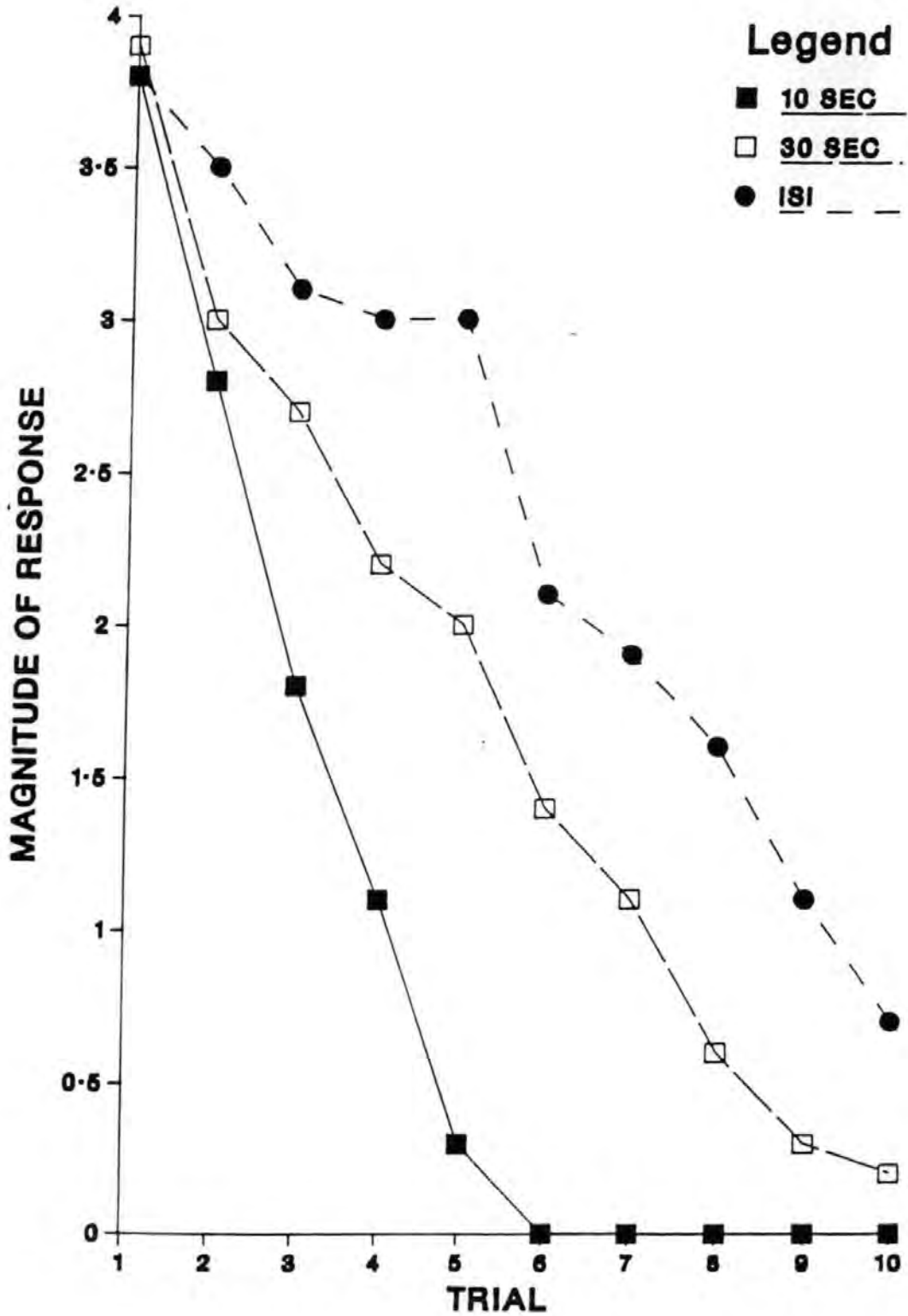
TABLE 5.2

TRIAL	DF	F VALUE	SIGNIFICANCE
1	2/27	0.44	$p > .5$
2	"	32.87	$p < .001$
3	"	120.09	$p < .001$
4	"	171.83	$p < .001$
5	"	109.95	$p < .001$
6	"	70.26	$p < .001$
7	"	29.85	$p < .001$
8	"	12.94	$p < .001$
9	"	10.08	$p < .001$
10	"	9.96	$p < .001$

The analysis of trials required to reach criterion of habituation within the confines of the fixed number of trials design revealed a significant difference between ISI conditions ( $F(2/27) = 68.831, p < .001$ ).

FIGURE 5.3

ISI AND DECREMENT IN RESPONSE MAGNITUDE





There was a significant difference between ISI conditions of 10 sec and 30 sec ( $F(2/27) = 151.005, P < .001$ ), the difference between ISI 30 and 60 sec failing to reach significance ( $F(2/27) = 1.345, P > .5$ ).

## DISCUSSION

The results of Experiment 2 agree with the relationship suggested in Experiment 1. Shorter ISIs produced smaller and shorter responses and faster habituation. Responses elicited at all stimuli presentation levels, except the first, varied as a function of ISI.

5:2                    ISI EFFECTS ON LONG TERM HABITUATION

### EXPERIMENT 3

The relationship between ISIs and long term habituation remains equivocal in both animal and human studies. No firm conclusions can be drawn from such a meagre and conflicting literature. However, although the evidence is somewhat inconclusive, there is some indication from these studies that long ISIs in training produce less recovery of the response in a long term test (see Chapter 2). Experiment 3 began a series of studies that investigated the effect of ISIs on long term habituation of the dorsal antennae withdrawal response in Helix aspersa. The effects of different ISI on both short and long term habituation are reported in this experiment with trials to habituation used

as a measure of habituation.

## METHOD

Subjects. Sixty mature snails were sampled from a laboratory housed population of 100 snails collected from the Plymstock area of Plymouth. Housing and criterion for participation was as reported in Experiment 1. Snails were numbered for purposes of identification using black paint. Indelibility of the numbers was ensured by varnishing the area of shell with the number on. The experiment was conducted on throughout April 1, 2, 3, 4, and 5, 1985, between the hours of 1830 and 0915. Temperature in the laboratory varied from 16 to 20 C.

Procedure. The participating snails were randomly assigned to one of 3 ISI conditions (20, 60 or 300 secs), with 20 snails per condition. All snails received 2 training sessions to criterion of habituation interspaced with a 12 hr inter-training interval. The technique for habituation and habituating stimulus were as reported in Experiment 2. Of the 20 snails in each ISI condition, 10 were given a retest for savings in terms of the number of trials to rehabituation 24 hrs after Session 2. The remaining 10 snails were given an identical retest 72 hrs after Session 2. ISI condition was maintained in both training sessions and during retest. Trials required to reach criterion in each session were recorded.

## RESULTS

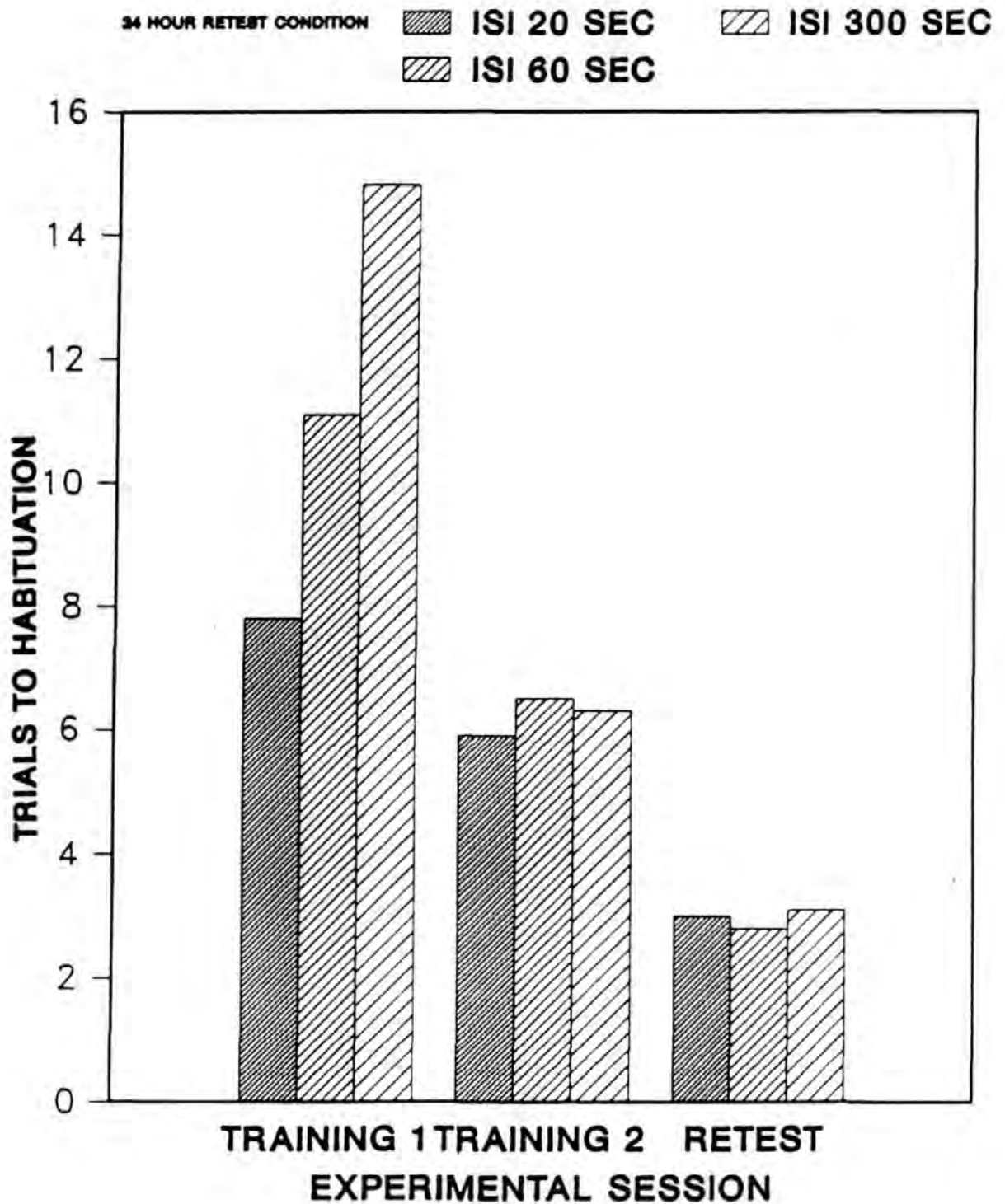
These data were analysed in a 3-way ANOVA with a within subjects factor of session and two between subject factors of ISI and retest. It revealed a significant effect of ISI ( $F(2/54) = 17.908, p < .001$ ) and session ( $F(1/54) = 482.993, p < .001$ ) but no significant difference between the two retests ( $F(1/54) < 1, p > .5$ ). There was a significant ISI x session interaction ( $F(2/54) = 43.853, p < .001$ ) and retest x session interaction ( $F(1/54) = 6.188, p < .025$ ), but neither the ISI x retest interaction nor the three way interaction of ISI x session x retest reach significance ( $F's(2/54) < 1$ ).

24 HOUR RETEST GROUPS. Analysis of simple main effects for snails retested 24 hrs post training revealed a significant decline in number of trials to criterion of habituation across Training Session 1, 2, and the retest for snails in the 20 sec ISI condition ( $F(1/54) = 61.19, p < .001$ ), the 60 secs group ( $F(1/54) = 181.05, p < .001$ ), and also in the 300 secs group, ( $F(1/54) = 382.86, p < .001$ ).

In all three ISI conditions greatest number of trials were required in Training Session 1, followed by Session 2 and least were required in retest (see Figure 5.4). All groups thus demonstrated long term habituation. Comparisons within each session across ISI conditions revealed a significant effect of ISI in training session 1 ( $F(2/54) = 96.296, p < .001$ ). Greatest number of trials were found to be required with the 300 sec ISI group ( $M = 7.8$ ), whereas, fastest habituation occurred in the 20 sec group ( $M = 14.8$ ). There was no effect of ISI in Session 2, or retest 24 hrs

FIGURE 5.4

THE EFFECTS OF ISI ON SHORT AND LONG TERM HABITUATION



after Session 2 (both  $F$ 's(2/54) < 1).

72 HOUR RETEST GROUPS. Analysis of simple main effects were then conducted for the 72 hr results. Again, there was a significant decline in trials to criterion within each ISI condition across sessions, 20 sec group ( $F(1/54) = 28.73$ ,  $p < .001$ ), 60 sec ( $F(1/54) = 145.24$ ,  $p < .001$ ), and 300 sec ( $F(1/54) = 355.74$ ,  $p < .001$ ). Most trials were required in session 1, least in the retest.




Analysis of each session across ISI's revealed a significant difference in short term habituation (Session 1) ( $F(2/54) = 116.398$ ,  $p < .001$ ). The number of trials required increased as ISI increased. There was no difference between groups 12 hrs later (Session 2) or at retest (72 hours) (both  $F$ 's < 1). The results are illustrated in Figure 5.5.

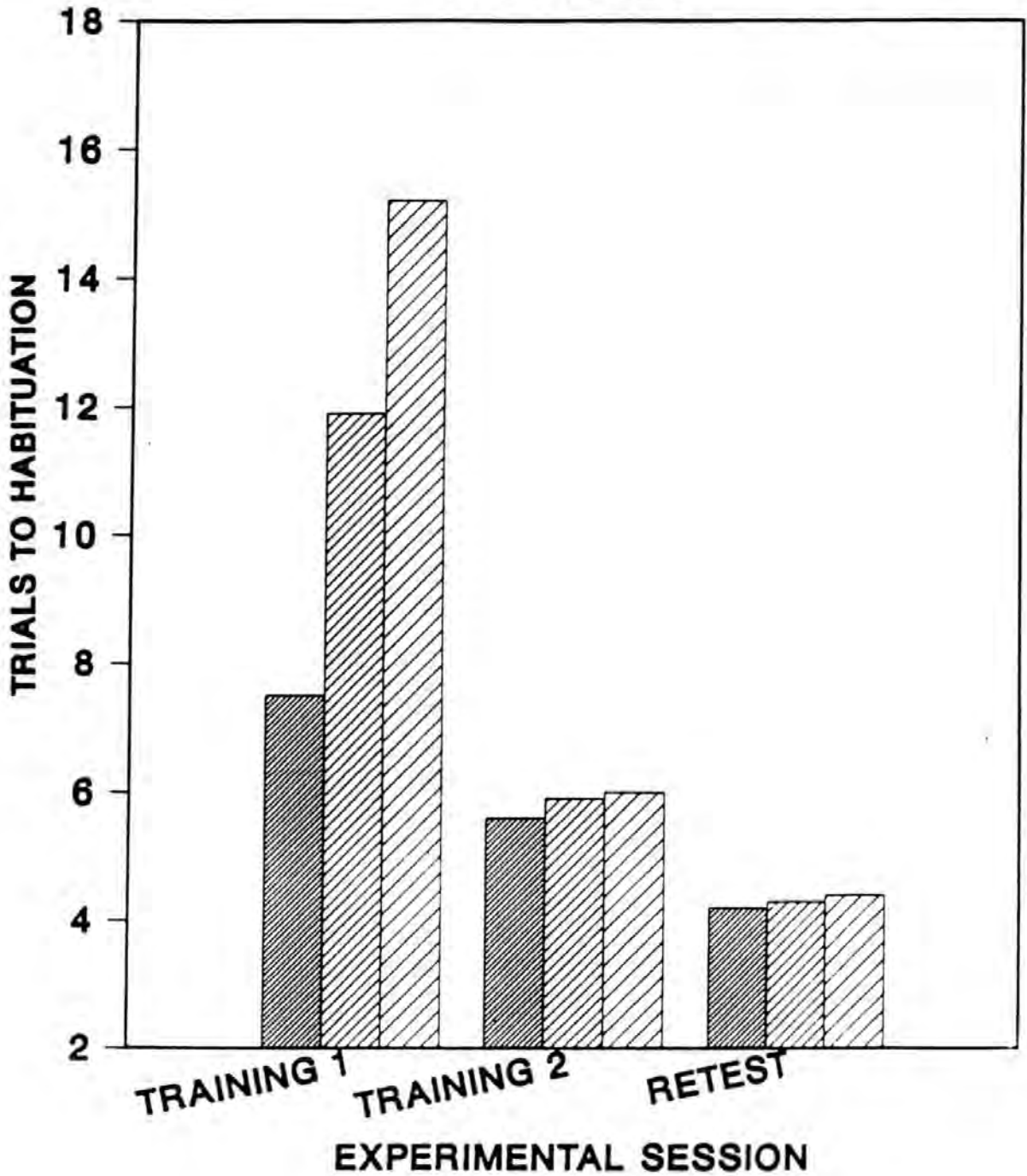
## DISCUSSION

As expected, in Session 1, shorter ISIs produced faster habituation. This is in accord with the findings of Experiments 1 and 2. In Session 2, 12 hrs after Session 1, the ISI effect was not apparent. Comparing savings in trials to habituation at session two with the number of trials required in the previous session, there was a saving in all ISI conditions. However, there was no difference in this saving between ISI conditions. ISIs had no effect on long term retention of habituation over 12 hrs. The relationship between ISI and short term habituation did not apply to long term habituation. Further evidence of this was provided by the retest data after the two training sessions. No

**FIGURE 5.5**

**ISI EFFECTS ON SHORT AND LONG TERM HABITUATION**

72 HOUR CONDITION     ISI 20 SEC     ISI 300 SEC  
                                ISI 60 SEC



significant difference was apparent between ISI conditions in either the 24 or 72 hrs retest. Both retest sessions showed evidence of retention in terms of savings, greatest savings being evident at the 24 hrs retest. However, there appears a differential effect of ISI on short and long term habituation. While in accord with the conclusion of Askew (1970), the results do not support the suggestion that long ISIs produce greater retention of habituation. There is no evidence of ISI effects in long term habituation once animals had been habituated to criterion. This measure of habituation may however mask ISI effects, as it is contaminated by short term, within session effects of ISI. Consequently, it could be argued that the long ISI conditions produced the same speed of habituation in the long term test despite the within session ISI effect. In order to obtain a measure of long term habituation effects which is not contaminated by within session effects, a further experiment was carried out.

#### EXPERIMENT 3(B)

Although it does have disadvantages (see introduction to this Chapter), Comparison of first response in each session as a measure of retention of habituation (Davis, 1970a) is uncontaminated by short term effects. This provides a second measure of the effects found in Experiment 3a. ISIs could conceivably produce effects on levels of responding in long term tests which would not be apparent with a trials to habituation measure. Consequently,

Experiment 3a was replicated utilising this second measure of long term habituation.

## METHOD

Subjects. Sixty snails were laboratory housed as reported in Experiment 1. All snails were collected from the Stonehouse area of Plymouth.

Procedure. Method and design were as reported in Experiment 3a. The experiment was conducted during April 10, 11, 12 and 13, 1985. Temperature in the laboratory varied between 16 and 19 C. Sixty snails were studied. Duration of first response was recorded for each of the two training sessions and in the retest session. Magnitude of response was also recorded for the same responses using the scale in Experiment 2. Habituation procedure and apparatus were as reported in the preceding experiment. The ISI conditions employed were again, 10, 60 and 300 secs.

## RESULTS

Data were analysed in a 3-way ANOVA with a within subject factor of session and between subject factors of ISI and retest interval. For response duration, there was a significant decrement of first response across session ( $F(1/54) = 740.882, p < .001$ ). There was no significant effect of ISI ( $F(2/54) < 1$ ) or retest interval ( $F(1/54) < 1$ ). Neither the ISI x retest, ISI x session, or retest x session interactions reached significance ( $F(2/54) < 1$ ). The ISI x



session x retest also failed to reach significance ( $F(2/54) < 1$ ). Greatest response durations were evidenced in Session 1, smallest in retest.

Category of response data was similarly analysed, a significant difference in response magnitude of first response across sessions ( $F(1/54) = 651.106, p < .001$ ), was revealed, with no significant effect of ISI ( $F(2/54) < 1$ ) or retest interval ( $F(1/54) < 1$ ). Similarly all interactions failed to reach significance ( $F(2/54) < 1$ ). Again the order of session effect revealed largest response magnitudes in Session 1, smallest in Session 2.

## DISCUSSION

Evidence of a decrement in first response over sessions was found. Long term retention at 12 hrs (in the second training session) and at both retest intervals of 24 and 72 hrs was evidenced. The level of savings in first response duration and magnitude did not vary as a function of ISI condition.

The results of these two experiments are not in accord with those of Askew (1970), who, essentially used a similar paradigm with one training session of five trials with a retest of five trials 30 mins later. Askew used the same ISI in retest as in training. Interestingly, the first response in the long term session of Askew's research was not affected by this confound, and here the efficacy of long ISIs in training was apparent. He reported large recovery of response during the 30 min inter training interval in the

1 and 10 sec groups but not in the 100 sec group, suggesting that longer ISIs in training produced less recovery of response in retest than short ISIs. Experiments 3a & b both failed to replicate this effect. While failing to replicate Askew's results, the above effects are in accord with Ron Ray's ISI data, who similarly found no long term ISI effects (see Chapter 2).

#### EXPERIMENT 4

Initial experiments thus far reported manipulate ISI in short term habituation but measured long term effects in a constant ISI retest. Davis (1970a) has criticised such an approach and suggested that the lack of long term ISI effects could be attributable to generalization, another parametric characteristic of habituation (see Chapter 2). In light of Davis's criticisms, a further experiment was conducted to replicate the results of Experiment 3a & b using the same ISI condition in training and retest within subjects and measuring ISI effects on speed of habituation and rehabituation.

#### METHOD

Subjects. Twenty snails were sampled from a population of 32 snails collected from a Plymouth garden and housed in a laboratory vivarium.

Procedure. The Experiment was conducted during May 6 and 7 1985. Temperature in the laboratory varied from 15 to

19 C. Criterion for participation in this experiment, habituation technique and stimulus were as reported in Experiment 1. Three ISI conditions were used, 10, 60, and 300 secs, to facilitate comparisons with Experiments 3a and 3b. Each snail received a training session to criterion and a retest 12 hrs post training. An ISI of 30 secs was employed in retest independent of original training ISI. The measure of habituation used was trials to criterion.

## RESULTS

The results of Experiment 4 mirrored the effects described in Experiments 3a & b and are presented graphically in Figure 5.6.

## DISCUSSION

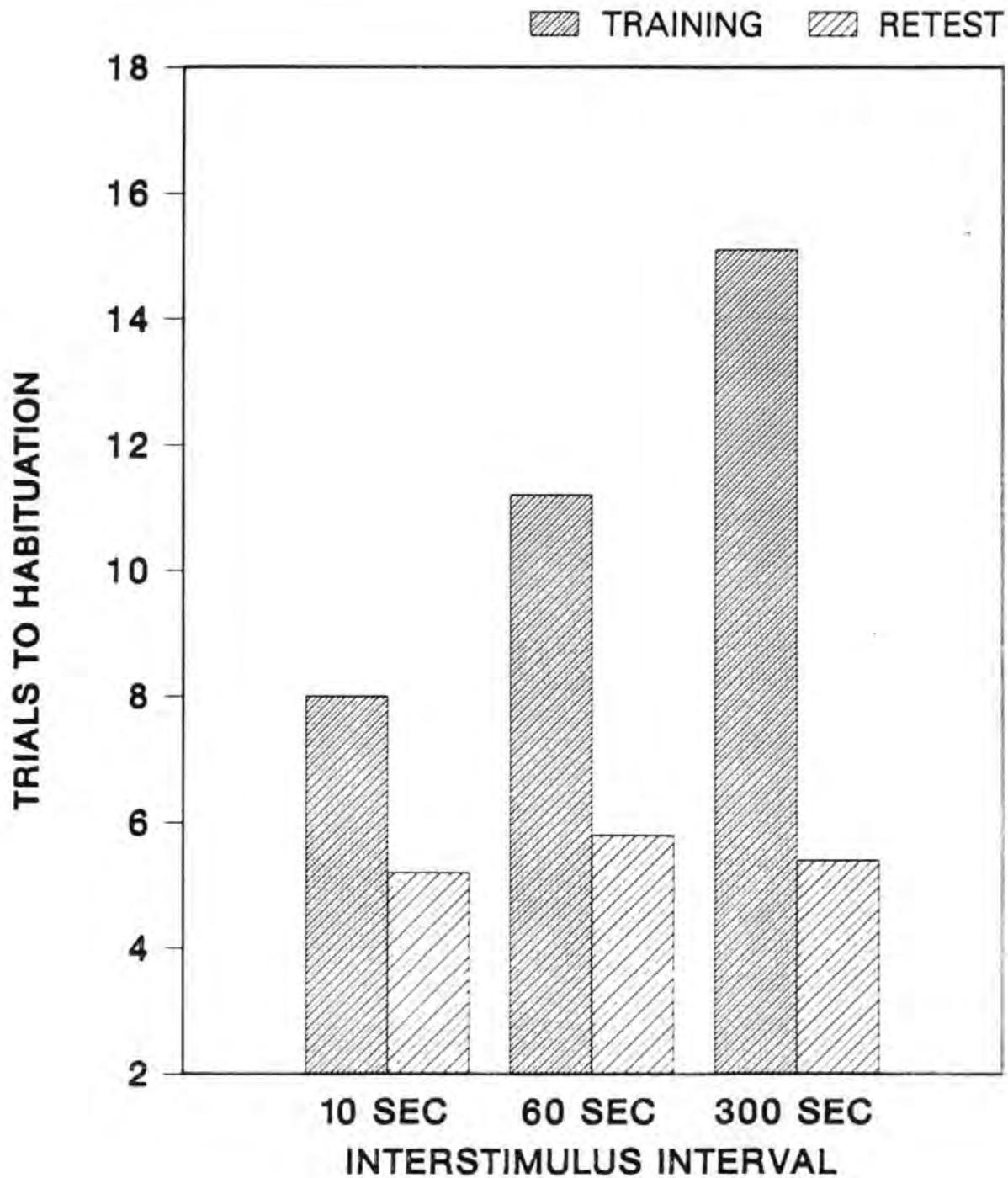
Results replicate the findings of Experiment 3a, negating the criticism of Davis, that using the same ISI in retest as in training could confound the long term test due to carry over effects (generalization) from the short term habituation. Long term habituation of the dorsal antennae withdrawal response in *Helix aspersa* showed again, no variation as a function of the ISI conditions.

## EXPERIMENT 5

A further long term study in the same preparation was conducted and used a fixed number of stimulus presentations

FIGURE 5.6

THE EFFECTS OF ISI ON SHORT & LONG TERM HABITUATION  
WHEN ISI REMAINED CONSTANT IN TRAINING AND RETEST



per session. Each response elicited to each stimulus presentation in two training sessions and a 48 hrs retest session was recorded.

Many of the studies of ISI effects on long term habituation report measures of habituation which only sample either changes in retest scores compared to training, or savings in initial response measures. Experiment 5 used a fixed number of trials approach, to avoid any potential confound caused by differential stimulus exposure between ISI conditions, and to quantify ISI effects of decrement of response in short and long term habituation.

#### METHOD

Subjects. Thirty snails were sampled from a population collected from the Stonehouse area of Plymouth.

Procedure. Housing and habituation technique was as reported in Experiment 1. Each snail received 2 training sessions of 10 stimulus presentations in one of of three ISI conditions 20, 60, and 300 secs. Each training session was interspaced by 12 hrs rest period. A retest was given 48 hrs after the second training session, and it comprised ten stimulus presentations. Duration of each response and magnitude of each response elicited were analysed in all sessions. Each ISI condition contained 10 animals whose participation was determined on the criterion developed in Experiment 1. Only in this experiment did this become an appreciable problem with 11 animals having to be replaced. The Experiment was conducted on December 3, 4, 5, and 6

between the hours of 0200 and 0930 hrs, and 1400 and 2100 hrs. Temperature in the laboratory varied from 15 to 19 C.

## RESULTS

Duration of response data were analysed in a 3-way ANOVA, with within subject factors of training session and response (trial) and a between subject factor of ISI. This revealed a significant effect of ISI ( $F(2/27) = 25.333$ ,  $p < .001$ ), training sessions ( $F(1/27) = 264.280$ ,  $p < .001$ ), and trials ( $F(1/27) = 578.971$ ,  $p < .001$ ). There were significant ISI x training session ( $F(2/27) = 26.334$ ,  $p < .001$ ), ISI x trial ( $F(2/27) = 5.366$ ,  $p < .001$ ), training session x trial ( $F(1/27) = 70.156$ ,  $p < .001$ ), and ISI x training session x response ( $F(2/27) = 6.230$ ,  $p < .01$ ) interactions.

Analysis of simple main effects demonstrated that in training session 1 shortest response durations were evidenced by snails in the 20 sec ISI condition, & largest in the 300 sec. This occurred at trials 2 to 9. Similarly there was a saving in response durations in subsequent sessions of habituation in all three ISI conditions. There was no ISI effect at Session 2 or the long term test (see Figure 5.7).

Category of response magnitude was analysed in the same way. Again, there was a significant effect of ISI ( $F(2/27) = 34.296$ ,  $p < .001$ ), training session ( $F(2/54) = 488.690$ ,  $p < .001$ ), and trial ( $F(1/27) = 17.961$ ,  $p < .001$ ), and there were significant ISI x training session ( $F(2/27) = 30.899$ ,  $p < .001$ ), ISI x trial ( $F(2/27) = 4.858$ ,  $p < .001$ ), training session x trial ( $F(1/27) = 17.961$ ,  $p < .001$ ), and ISI x

FIGURE 5.7a

ISI AND RESPONSE DECREMENT IN TRAINING 1

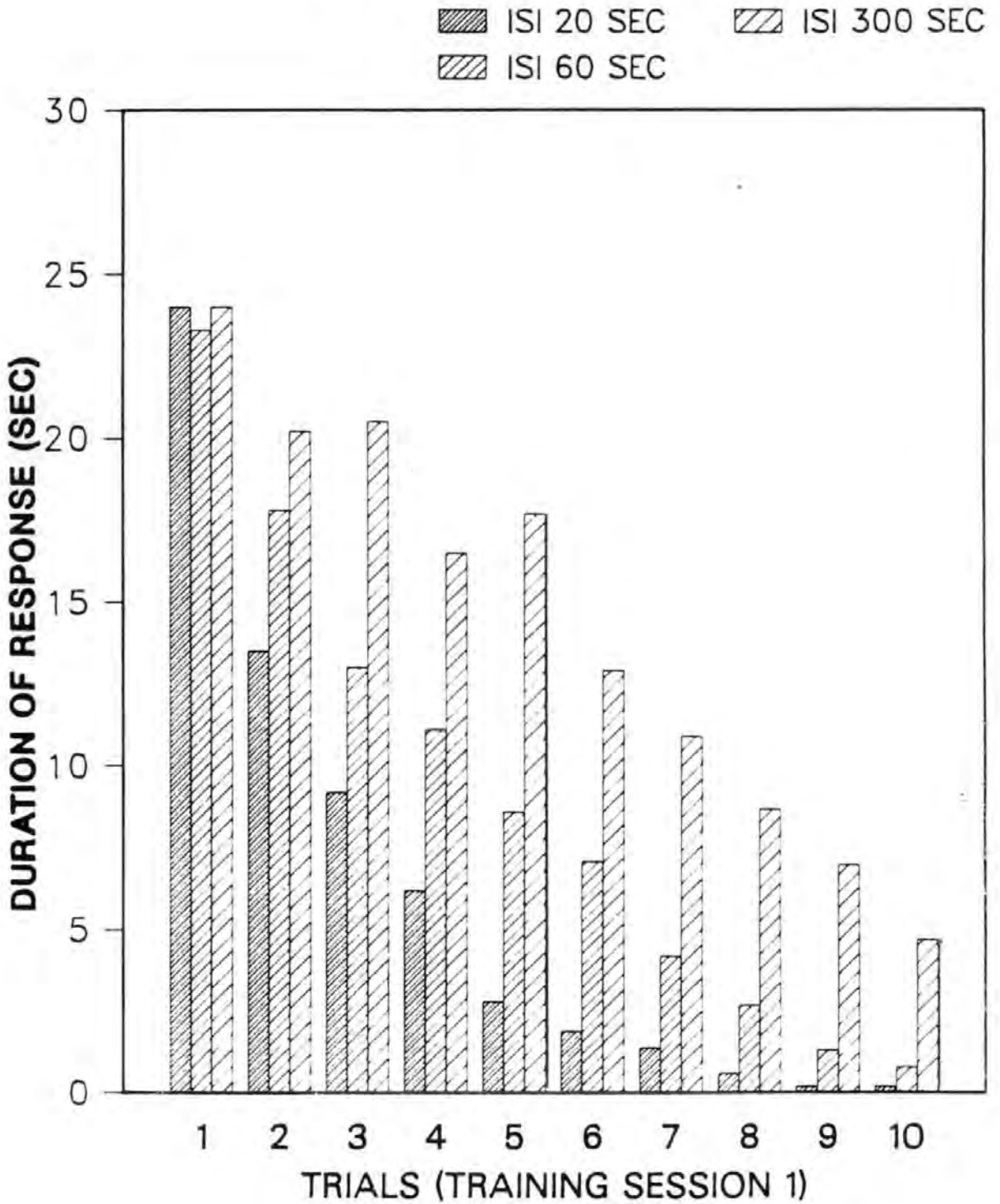


FIGURE 5.7b

ISI AND RESPONSE DECREMENT IN TRAINING 2

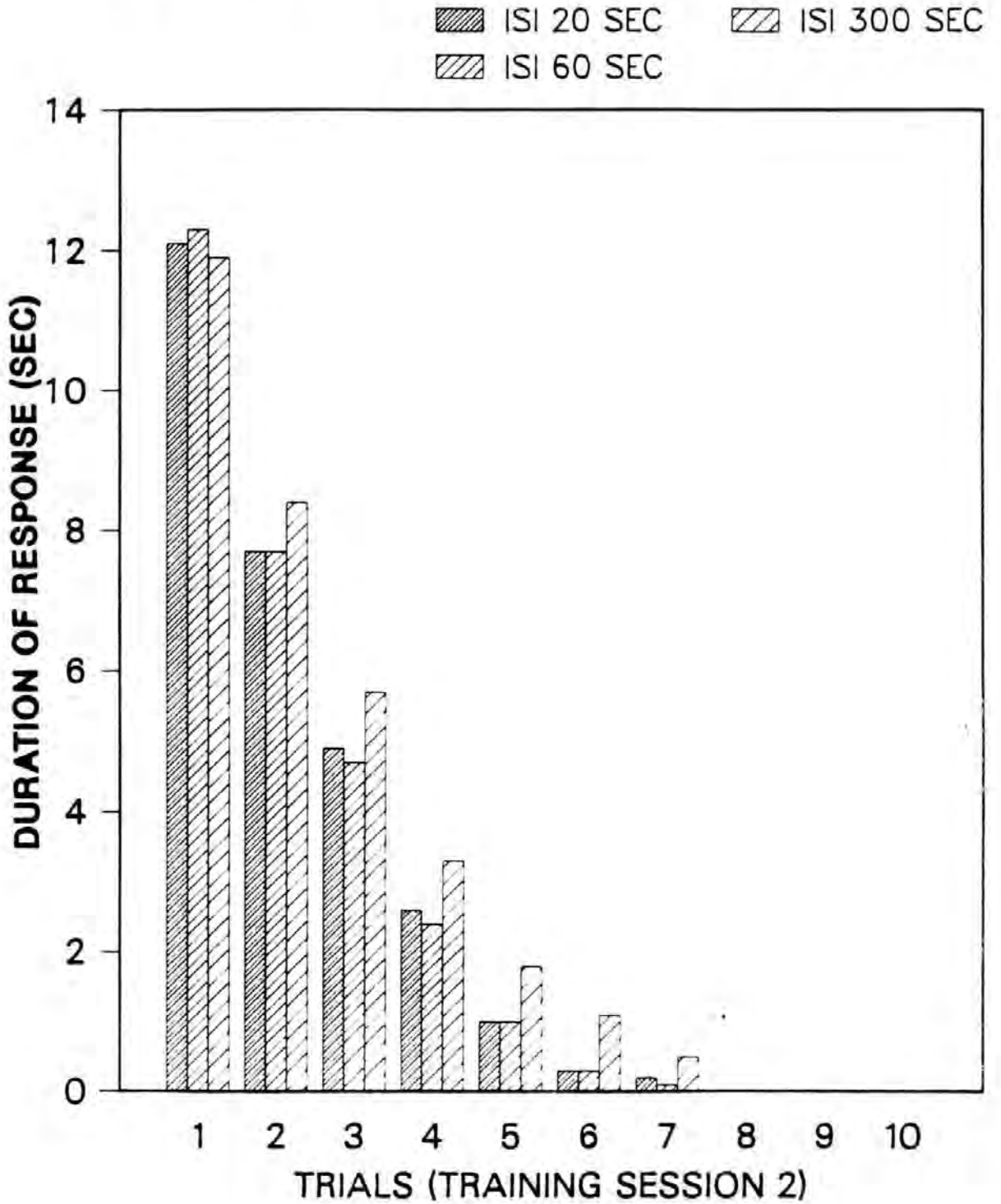
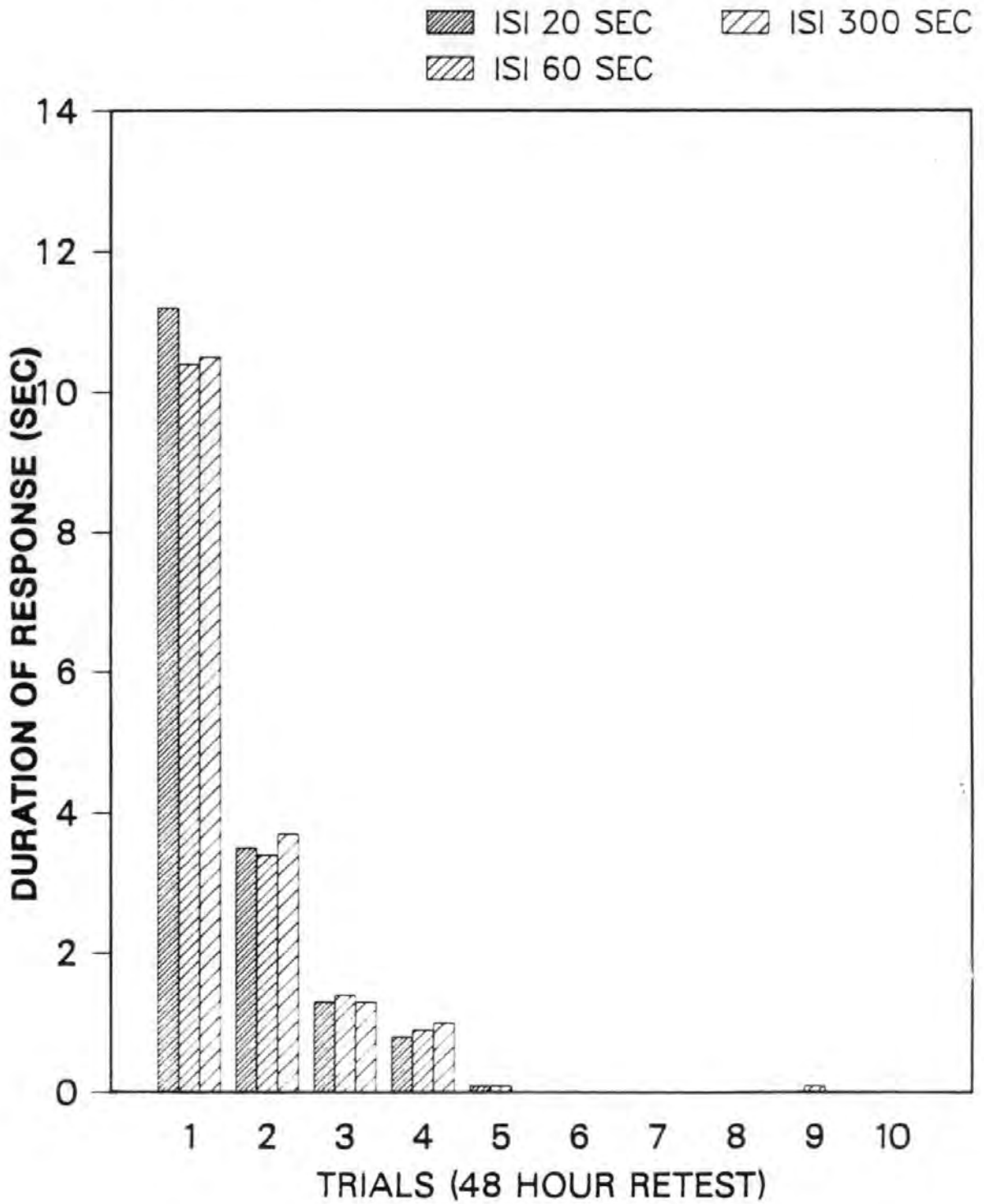




FIGURE 5.7c

ISI AND RESPONSE DECREMENT IN RETEST (48 HRS)



training session X trial( $F(2/27) = 5.812, P < .01$ ) interactions.

Analysis of simple main effects revealed the same findings with those reported for the response duration data reported above (see Figure 5.8). A decrement in response over iterated trials was found in all ISI conditions in all sessions. There was no effect of ISI in Session 2 or retest. In initial training shorter ISIs produced successively smaller responses which suggests greatest habituation in the short ISI conditions.

## DISCUSSION

The results from Session 1 were in agreement with those from Experiment 2, namely, short ISIs produced shorter and smaller responses across trials. There was no ISI effects in the second training session. This in itself could be considered a test of long term habituation, and it indicates that ISI has no effect on long term habituation. This conclusion is supported by results from the 48 hrs retest where, again, no ISI effects were evidenced.

## GENERAL DISCUSSION

In the habituation of the dorsal antennae withdrawal response of the snail, short ISIs produce faster short term habituation than long ISIs and produces smaller and shorter responses across trials. This does not appear to be the case for long term habituation.

FIGURE 5.8a

ISI AND DECREMENT IN RESPONSE MAGNITUDE

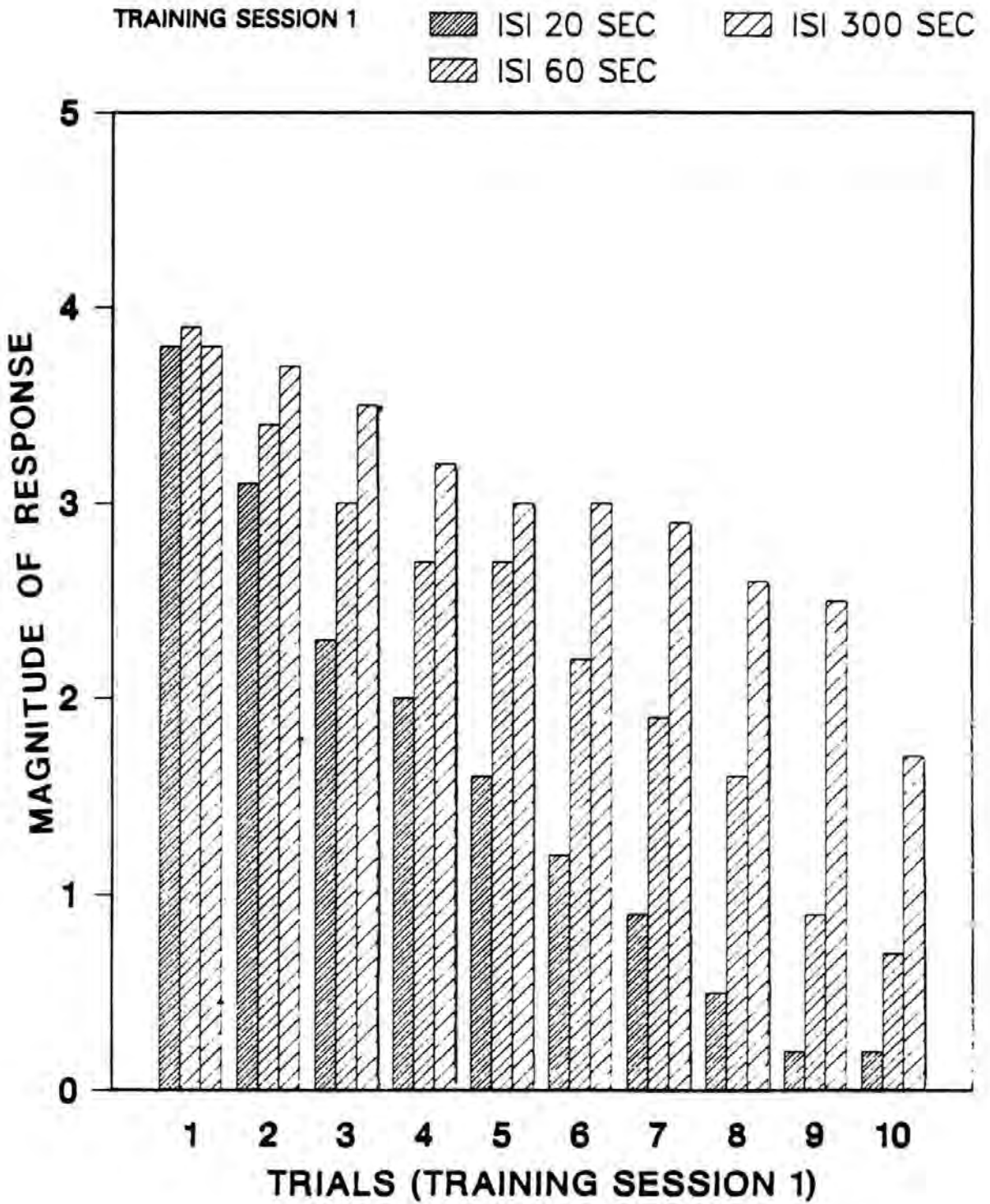


FIGURE 5.8b

ISI AND DECREMENT IN RESPONSE MAGNITUDE

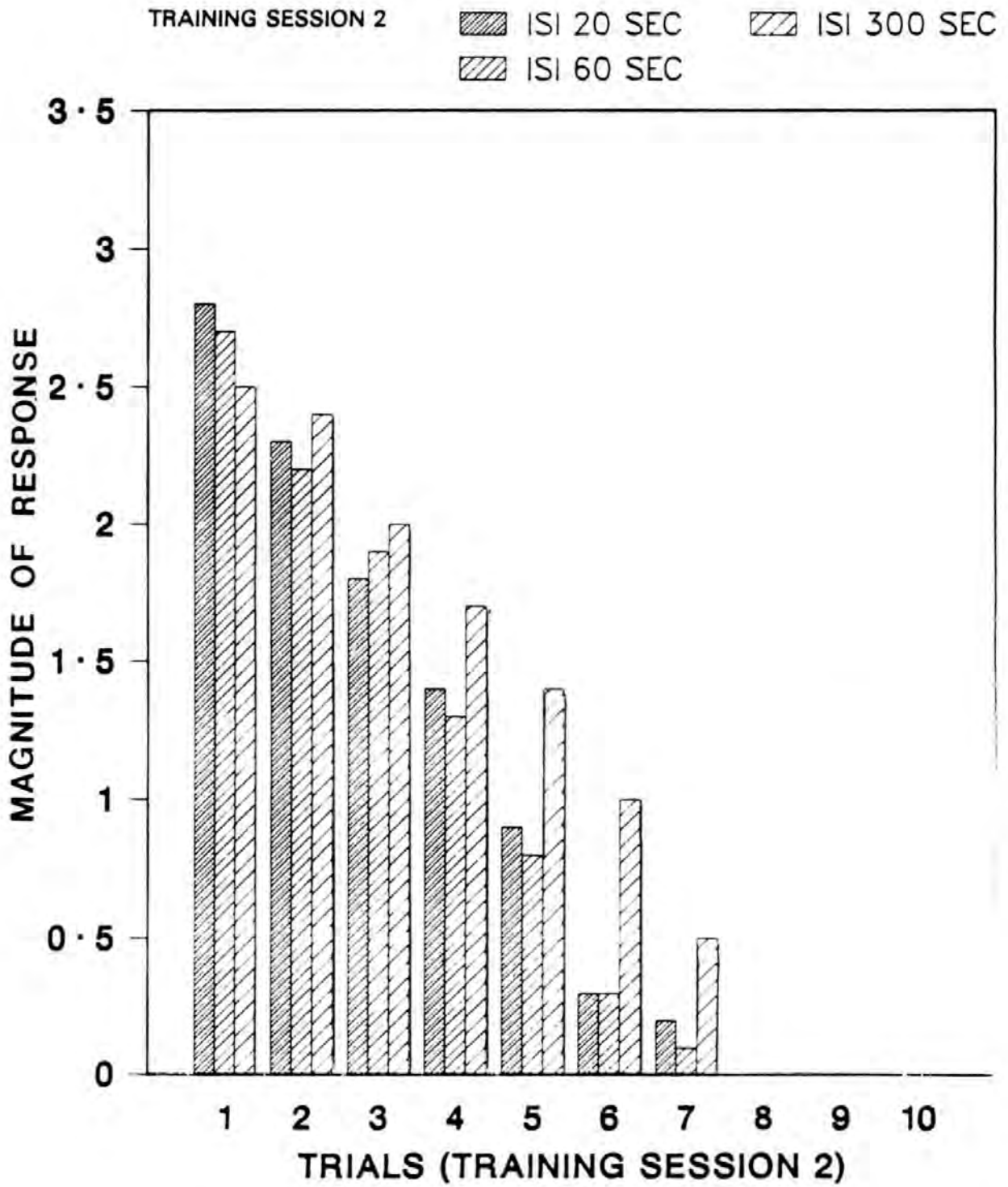
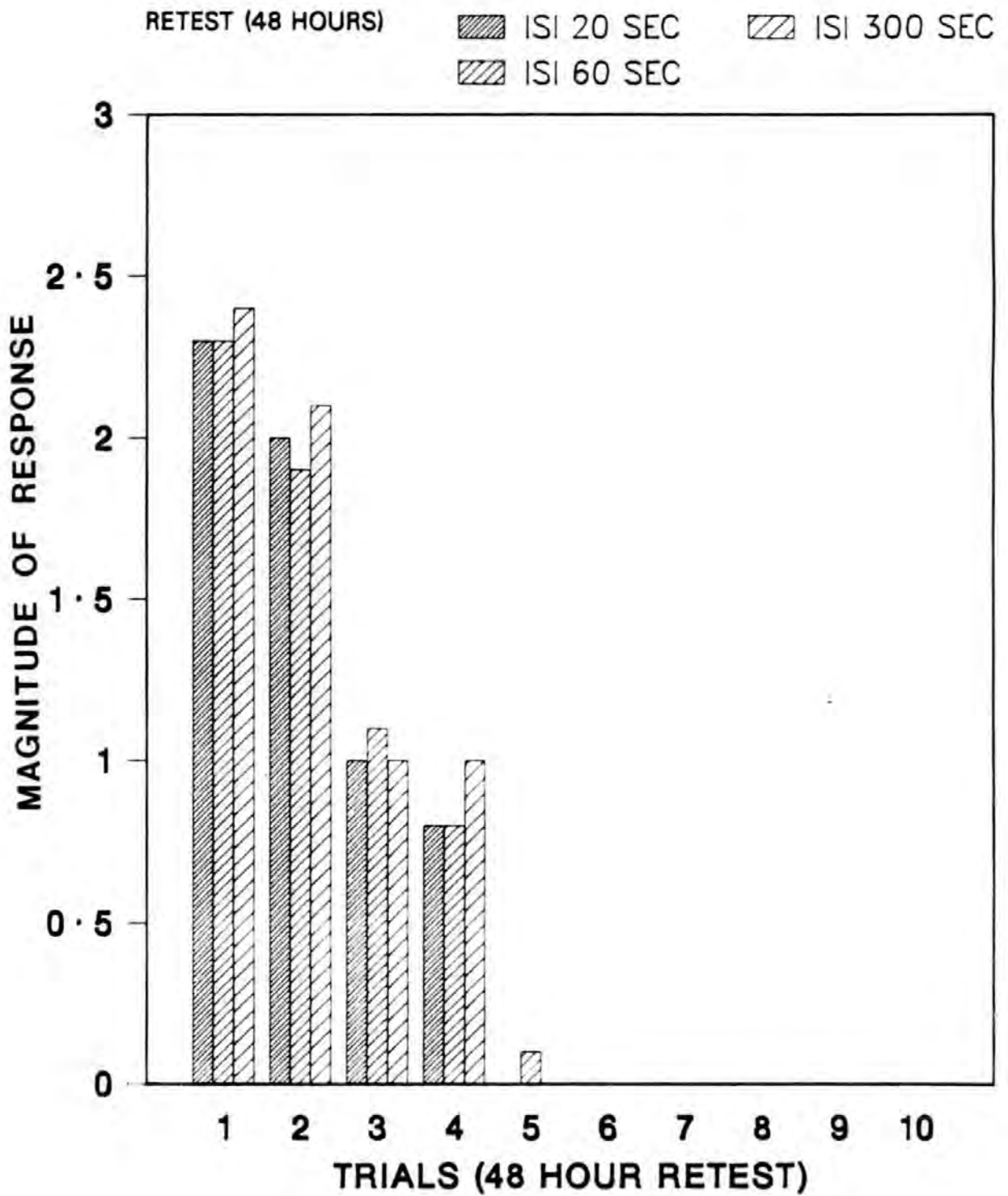


FIGURE 5.8c

ISI AND DECREMENT IN RESPONSE MAGNITUDE



These results are in accord with the view that short and long term habituation are different in that there are differences in their parametric characteristics (e.g. Askew, 1970; Buchwald & Humphrey, 1973; Castelluci & Kandel, 1976; Davis, 1970a,b; Graham, 1973; Groves & Lynch, 1972; Hinde, 1954; Kohler, 1976; Leaton, 1976; Ohman, 1979; Peterson & Squire, 1977; Sharpless & Jasper, 1956; Sokolov, 1955, 1969; Wagner, 1976, 1978, 1979, 1981; Whitlow & Wagner, 1984). What is more, the experiments reported in this chapter used a diversity of measures of habituation, and all measures produced results in agreement with the conclusion that shorter ISIs do produce faster short term habituation but there is no long term effect. These results demonstrate that habituation in *Helix* shares the same short and long term features of habituation demonstrated in other species. These results are of some importance to theories of habituation. For example Carew et al. (1972) suggested a mechanism for the superiority of short ISIs over long ISIs in short term habituation. They proposed that with short ISIs, faster firing of the reflex neuronal pathway produces more c-AMP which, in turn, phosphorylates gating proteins such that the threshold of the pathway is raised. They similarly suggest that superior long term habituation would result from longer ISIs. Their mechanistic account for this conclusion is less extensive and is discussed in the next chapter.

Carew et al. discuss the above effects based on research using a different distribution of training rather than manipulating ISI within sessions per se. Indeed ISI is

not the only way of distributing training. Consequently, a further series of experiments were conducted to investigate other forms of massing and spacing of training on short and long term habituation in this response.

## CHAPTER 6

### THE EFFECTS OF MASSED AND SPACED TRAINING ON SHORT AND LONG TERM HABITUATION

There are many other ways of distributing learning other than varying ISI. Indeed, one of the early, behavioural experiments which had an influence on Kandel's theorising involved differential massing and spacing of practice with *Aplysia* (Carew et al., 1972).

Investigations of massing and spacing of practice other than ISIs have employed a variety of forms of distributions of practice. Those studies which have compared training on one day with that spaced over several days (Carew et al., 1972; Fearing, 1940, 1941; Hood and Pfaltz, 1954) and training massed into one series of trials with that spaced over several series within one day (Dyal and Hetherington, 1968) or across sessions separated by different number of days (Brown, 1965), have consistently reported no effect on short term habituation but less recovery of the response in the long term after spaced training than after massed training. However, Danziger and Mainland (1954) reported that massed training was more effective than spaced training in producing short term response decrement, whereas, Peeke and Peeke (1970) reached the opposite conclusion. The situation is still further confused since Brown and Noakes (1974), with a similar distribution of training, found no difference between relatively massed and spaced conditions, but both were more effective than continuous exposure (see



Chapter 2).

Comparisons between experiments and different forms of massing and spacing of training is further confounded by a lack of standardisation in measures of habituation used and the variety of animals used.

A series of 3 experiments are reported in this chapter which explore the effects of massed and spaced training on short and long term habituation of the dorsal antennae withdrawal response in the snail using measures of both short and long term habituation established in earlier chapters.

#### EXPERIMENT 1

This experiment investigates the differential effects of within session massing and spacing of training on short and long term habituation. Speed and retention of habituation was measured in terms of trials required to reach habituation criterion (see Experiment 1, Chapter 3).

#### METHOD

Subjects. Forty mature Helix aspersa from a population of 63 snails collected from the Bovisand Bay area of Plymouth (UK.) were sampled. All snails were laboratory housed in a glass vivarium (50 x 50 cm) which contained a grass turf and loose vegetation removed from the area of capture. The diet of the snails was supplemented by 12 oz of fresh lettuce leaves placed in the vivarium at 48 hr

intervals. All snails were numbered using black paint covered with a coat of clear marine varnish.

Procedure. Data collection commenced one week from the night of capture (June 5, 1985). The experiment was conducted between 1600 and 1030 overnight on June 12, 13, 14, and 15, 1985. Temperature in the laboratory varied between 19 and 21 C. Ten snails were assigned randomly to one of four experimental conditions. Two groups received massed training, and two groups received spaced training. The training stimulus and presentation were the same as used in Experiment 1, Chapter 3. In the massed training groups, snails were repeatedly presented with the tactile stimulus at a constant ISI of 30 secs until criterion of habituation was reached. In the spaced training conditions snails received two stimulus presentations spaced by 30 secs followed by a further 4 mins rest then two more presentations interspaced by 30 secs. This procedure was repeated until criterion of habituation was reached.

Prior to the commencement of a training session, the snail was removed from its home vivarium and gently placed on a varnished wooden test-board, 30 x 30 cm. It then received a 1 min 'apparatus orientation period' in which it remained unmolested. If, after this period, the snail was fully extended from its shell and active, the snail participated in the experiment. Participation was terminated if, at any stage of training or retest, it withdrew into its shell for a sufficient duration that it missed a scheduled stimulus presentation, or if it crawled off the board. All such subjects were replaced with animals from the same

captive population. Three such substitutions were necessary. Snails remained on the test apparatus during all ISIs. At the end of the training session, the snail was returned to its home vivarium to await its scheduled retest. Two retest intervals were employed as a between subjects factor; Namely, 12 and 24 hrs post-training. A second apparatus orientation period preceded retest, with the same criterion for the snails continued participation. All interstimulus and interblock intervals were timed from the termination of preceding response.

Habituation procedure at retests was constant with repeated presentations of the tactile stimulus at a regular ISI of 30 secs irrespective of the snails initial training. This consistent retest paradigm was utilised to avoid any confound in the long term habituation test from variance in retest procedure. All retesting of animals was conducted using a blind procedure, snails due for retest were passed by an assistant with their identification numbers masked with tape, and the assistant recorded the snails' training profile.

## RESULTS

Data were analysed in a mixed design, 3-way ANOVA, with training (massed/spaced) and retest interval as between subject factors and session (training/retest) as the within subject factor. There was a significant effect of training condition ( $F(1/36) = 5.16, p < .05$ ), retest interval ( $F(1/36) = 7.561, p < .01$ ) and session ( $F(1/36) = 266.912, p < .001$ ).

There were the following interactions; training condition x session ( $F(1/36) = 14.912, p < .001$ ), retest interval x session ( $F(1/36) = 14.912, p < .001$ ) and training condition x retest interval x session ( $F(1/36) = 4.324, p < .05$ ). These results are illustrated in Figure 6.1.

Analysis of simple main effects revealed that snails tested at 12 hrs post training, exhibited no significant difference between massed and spaced training in short term habituation ( $F(1/36) = < 1$ ). However, snails in the massed group required more trials to habituation at retest than those in the spaced condition ( $F(1/36) = 6.17, p < .025$ ). There was a saving in trials to criterion at this retest in both massed ( $F(1/36) = 84.789, p < .001$ ) and spaced conditions ( $F(1/36) = 120.79, p < .001$ ).

Snails retested at 24 hrs post training again showed no difference in the first training session ( $F(1/36) = < 1$ ). However, there was a difference between training condition at retest ( $F(1/36) = 28.587, p < .001$ ). Spaced trained snails required fewer trials to reach criterion than massed trained at this retest. Both training conditions resulted in a saving in trials to habituation at retest. Greatest saving was evidenced by snails in the spaced condition ( $F(1/36) = 84.789, p < .001$ ), but savings in massed condition still reached significance ( $F(1/36) = 10.676, p < .01$ ). In both training conditions, there was more retention in the 12 hr test than evidenced in the 24 hr test ( $F(1/36) = 29.789, p < .001$ ). These results are shown in Figure 6.1a.

**FIGURE 6.1**

**THE EFFECTS OF MASSED AND SPACED TRAINING ON SHORT AND LONG TERM HABITUATION**

RETEST 12 HOURS

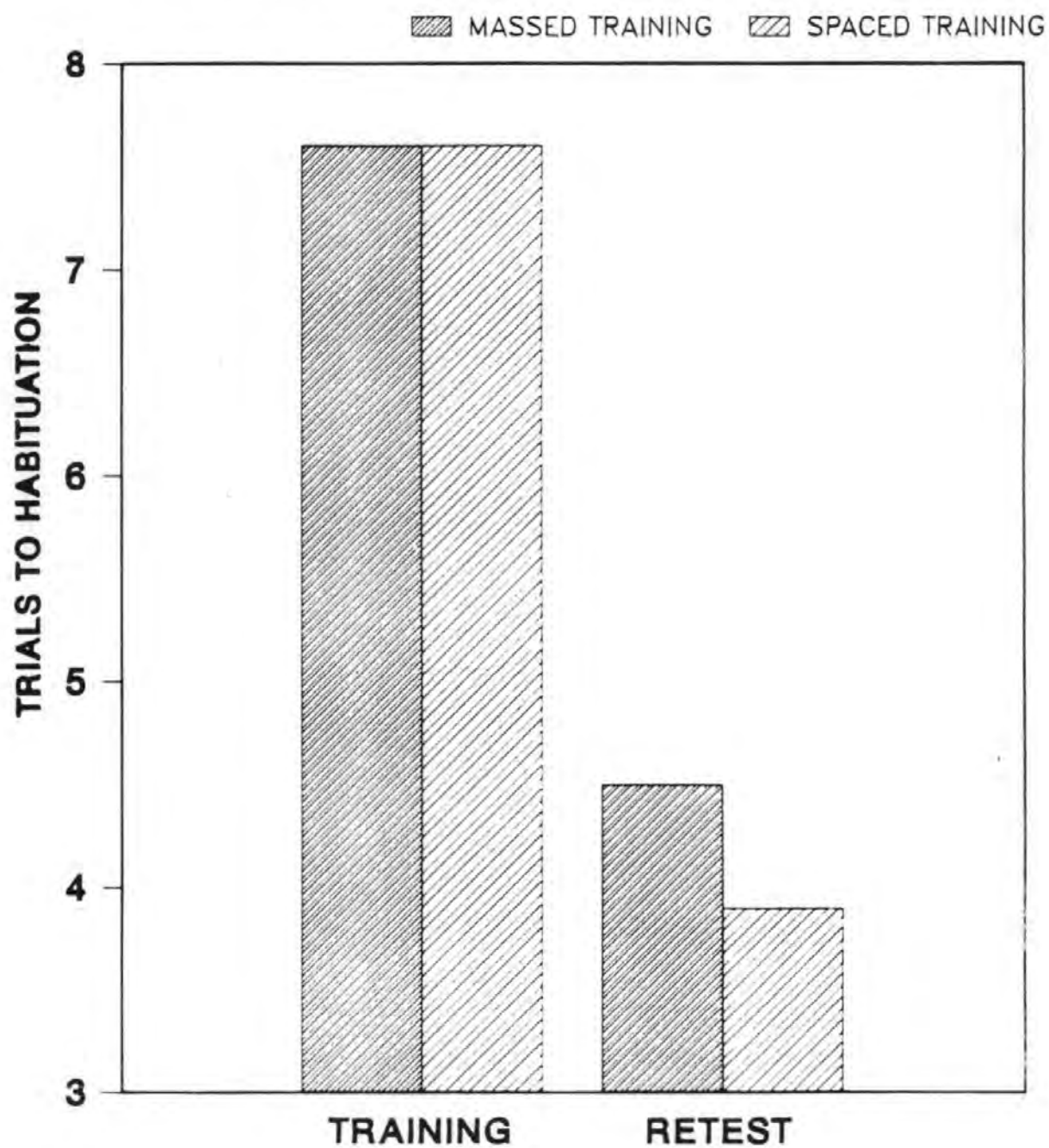
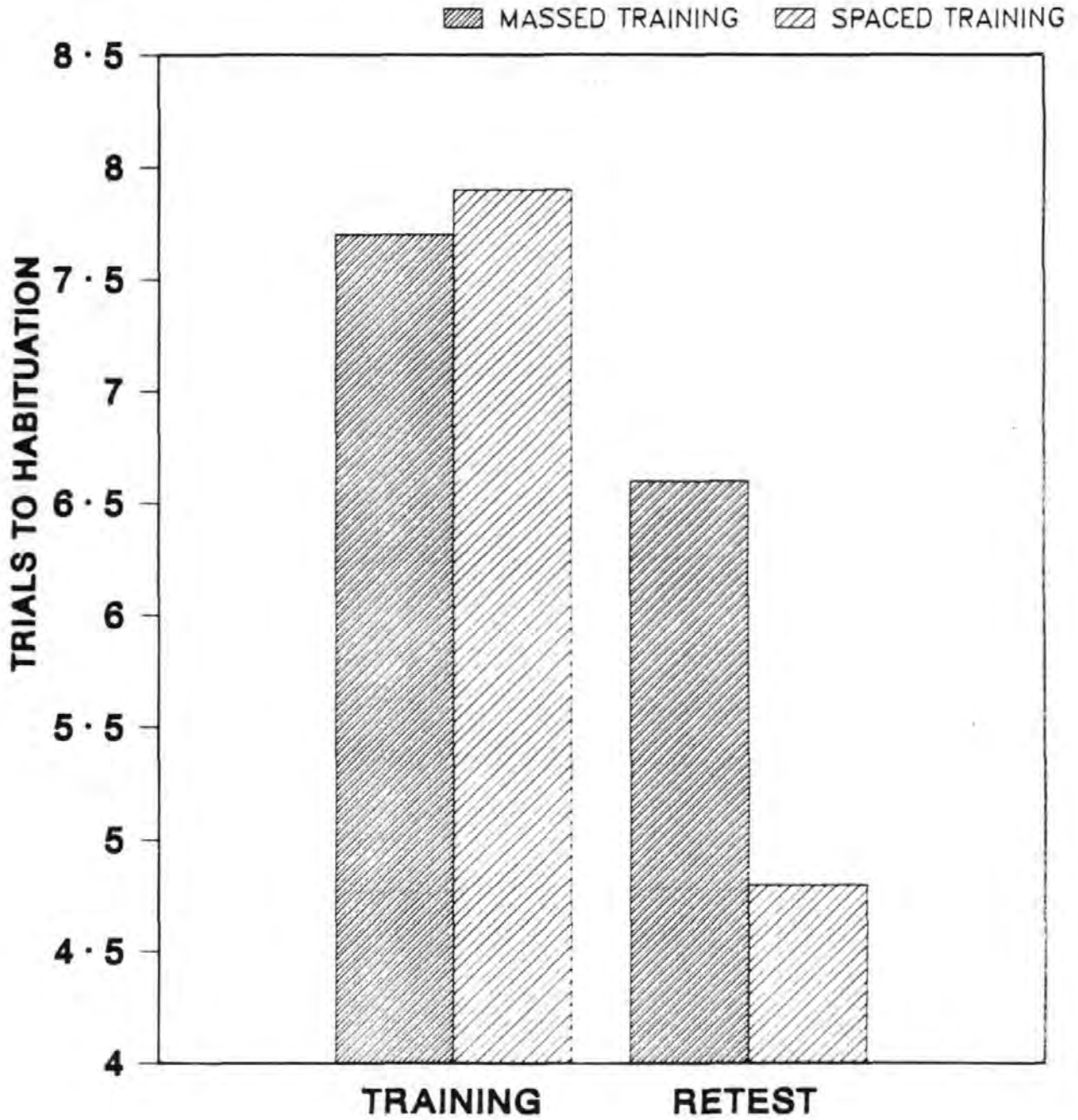


FIGURE 6.1a

THE EFFECTS OF MASSED AND SPACED TRAINING ON SHORT AND LONG TERM HABITUATION

RETEST 24 HOURS



## DISCUSSION

There appeared to be no <sup>differential</sup> effect of massed and spaced training on short term habituation. All snails required approximately the same number of trials to reach habituation criterion. However, analysis of long term habituation showed superior retention after spaced training.

## EXPERIMENT 2

As there were no effects in short term habituation in Experiment 1, the problem of differential stimulus exposure (see Experiment 5, Chapter 5) does not arise. However, in order to replicate the above findings, a fixed number of trials experiment was conducted to investigate any subtle effects massing and spacing of training may have on the development of short and long term habituation.

## METHOD

Subjects. Twenty mature Helix aspersa from a population of 46 snails captured in the Princetown area of Dartmoor (UK.). All snails were selected on the same criterion for participation as that reported in Experiment 1.

Procedure. The experiment was conducted on June 16, 17 and 18, 1985. Temperature in the laboratory varied from 16 to 21 C. Laboratory housing and training stimulus and procedure were as reported in the previous experiment, with

the exception that all snails received a fixed number of stimulus presentations (10) rather than training to criterion. All responses elicited were measured for duration and magnitude (see Chapter 3)

Ten snails were randomly allocated to each of two experimental training conditions; spaced or massed practice. Distributions of practice in these two conditions were the same as those reported in Experiment 1.

All retests consisted of 10 repeated presentations of the same training stimulus with ISIs of 30 secs and commenced 24 hrs after termination of training.

## RESULTS

The acquisition and retention curves for both massed and spaced conditions are depicted in Figure 6.2 a & b, and 6.3 a & b.

Both response duration and magnitude were analysed in a 3-way ANOVA with a between subjects factor of training condition and within subject factors of session and trial. Response duration data revealed a non significant effect of training condition ( $F(1/18) = <1$ ), a significant effect of session ( $F(1/18) = 52.87, p < .001$ ), and trial ( $F(1/18) = 501.884, p < .001$ ) with significant interactions between training condition and session ( $F(1/18) = 6.599, p < .025$ ), and session x trial ( $F(1/18) = 28.203, p < .001$ ). All other interactions failed to reach significance ( $F(1/18) < 1$ ).

Analysis of simple main effects revealed there to be no significant difference between massed and spaced training in



**FIGURE 6.2a**

THE EFFECTS OF MASSED AND SPACED TRAINING  
ON SHORT TERM HABITUATION

TRAINING

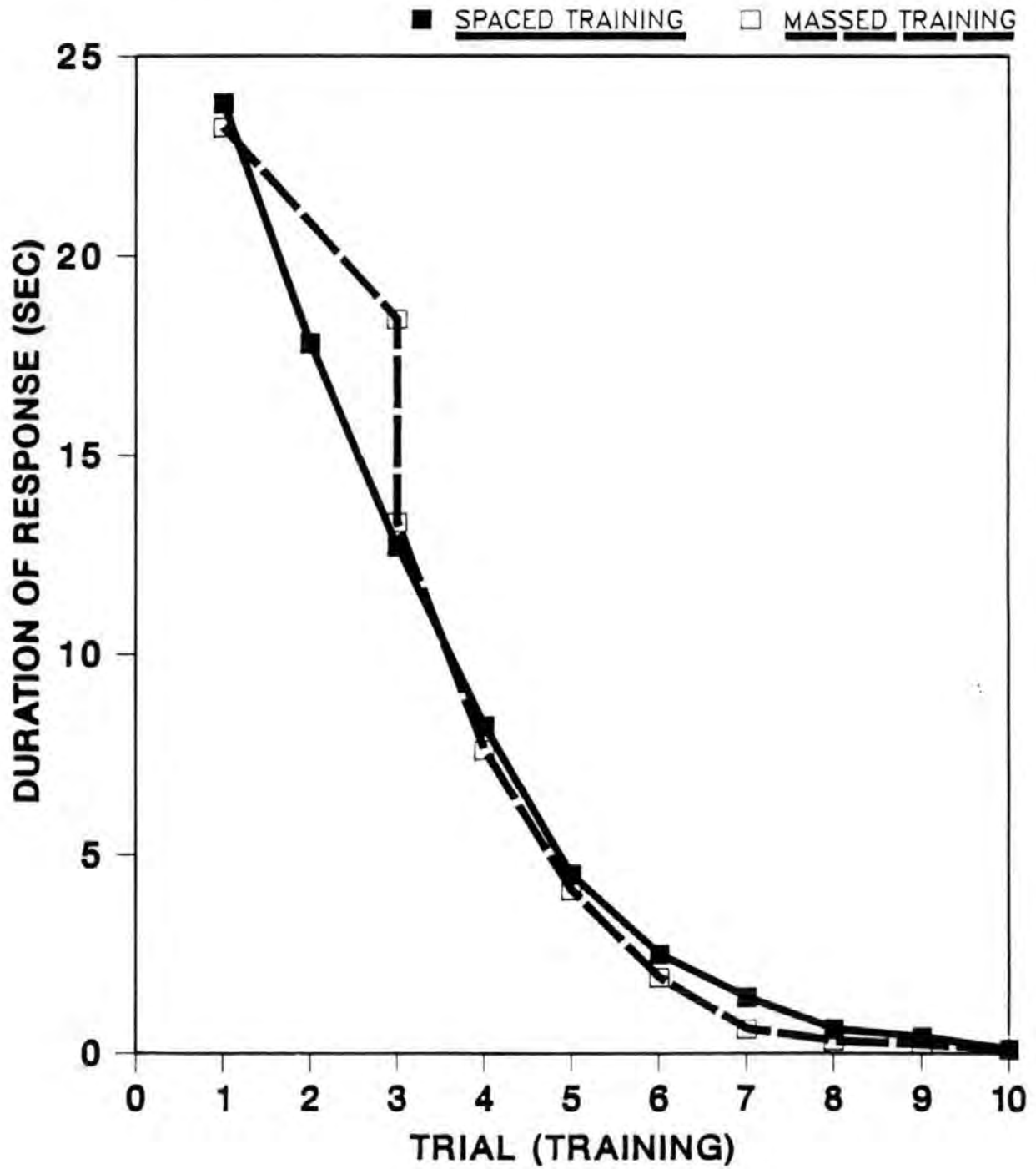


FIGURE 6.2b

THE EFFECTS OF MASSED AND SPACED TRAINING  
ON LONG TERM HABITUATION

RETEST

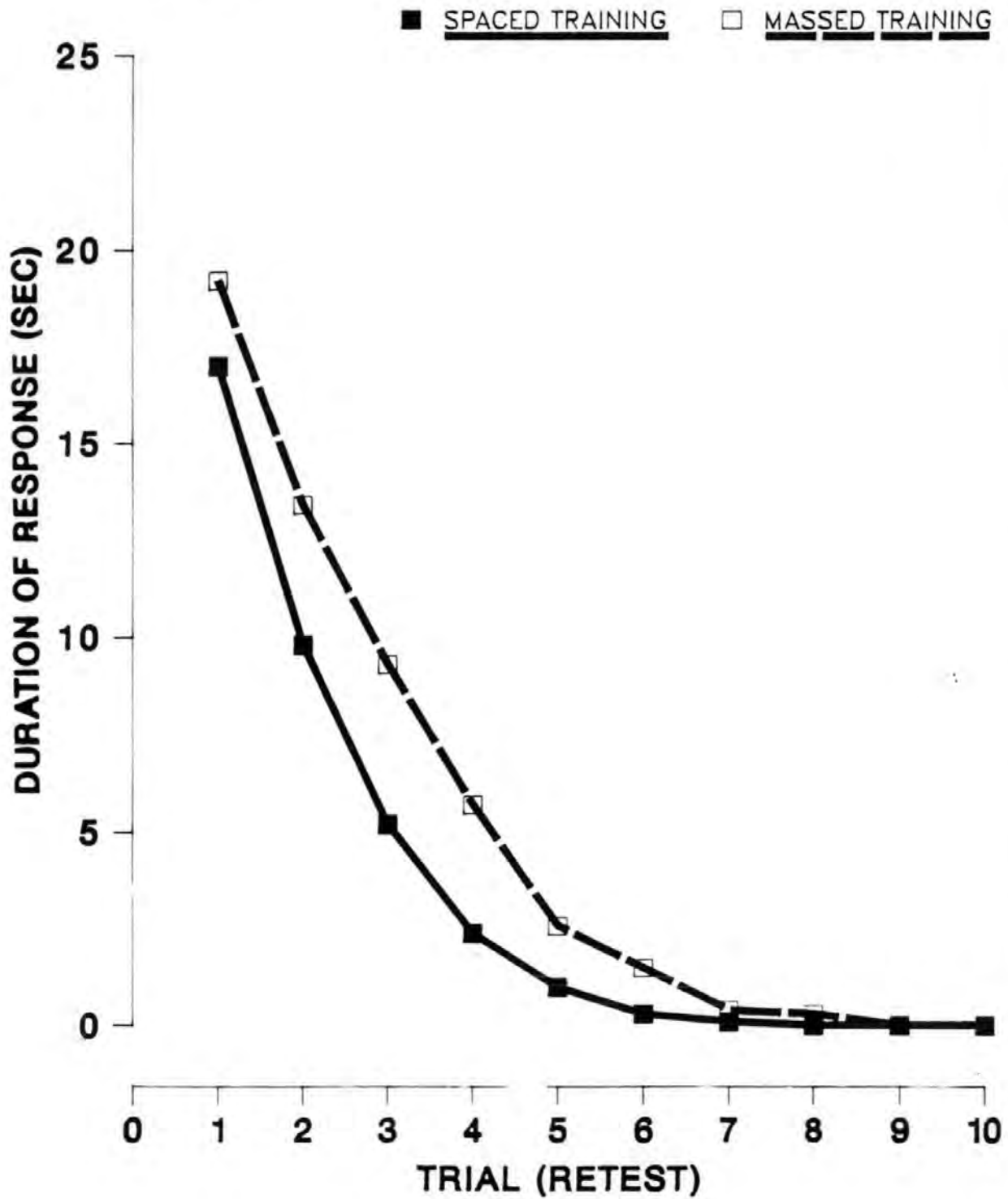


FIGURE 6.3a

EFFECTS OF MASSED AND SPACED TRAINING ON SHORT TERM HABITUATION (RESPONSE MAGNITUDE DATA)

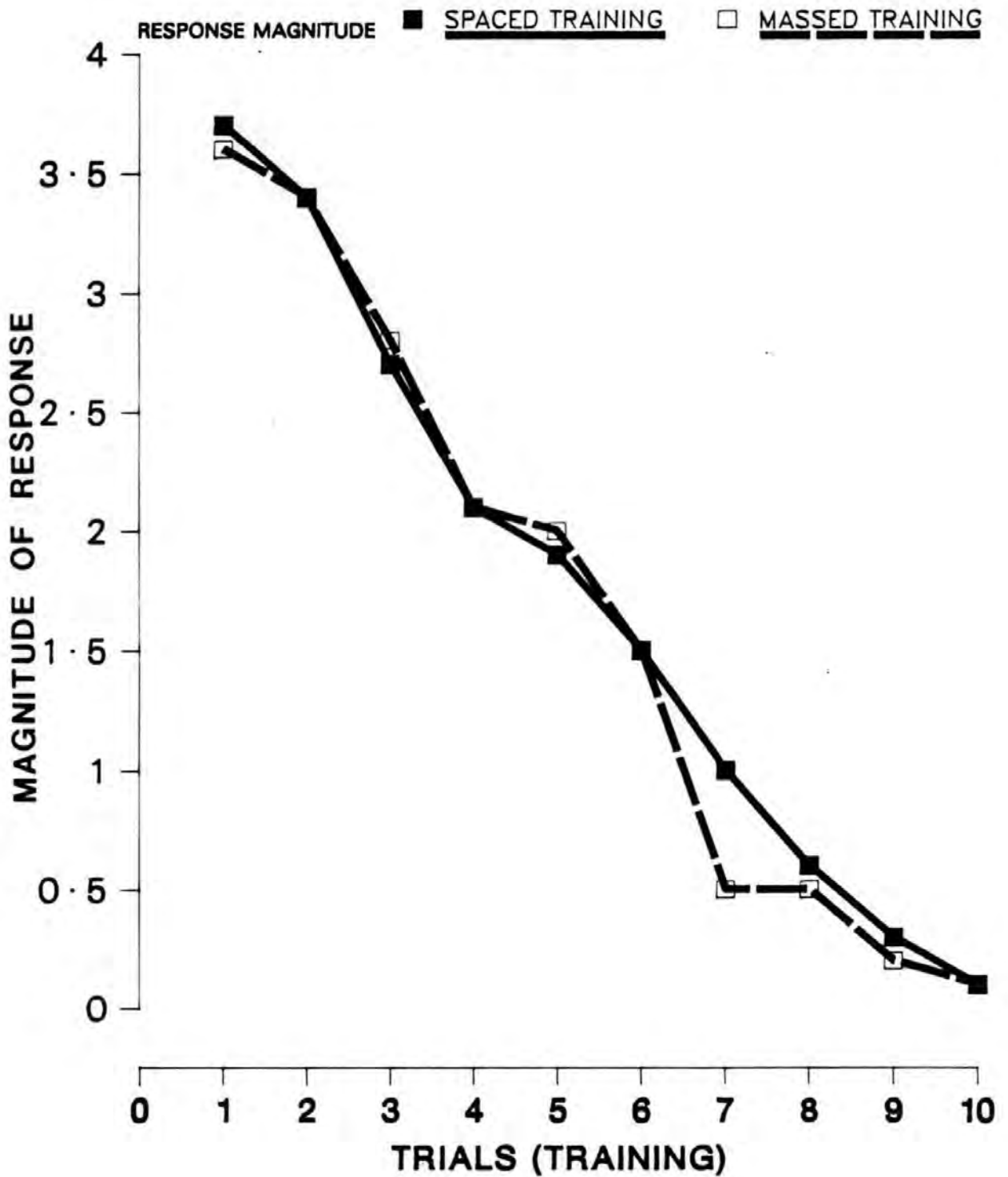
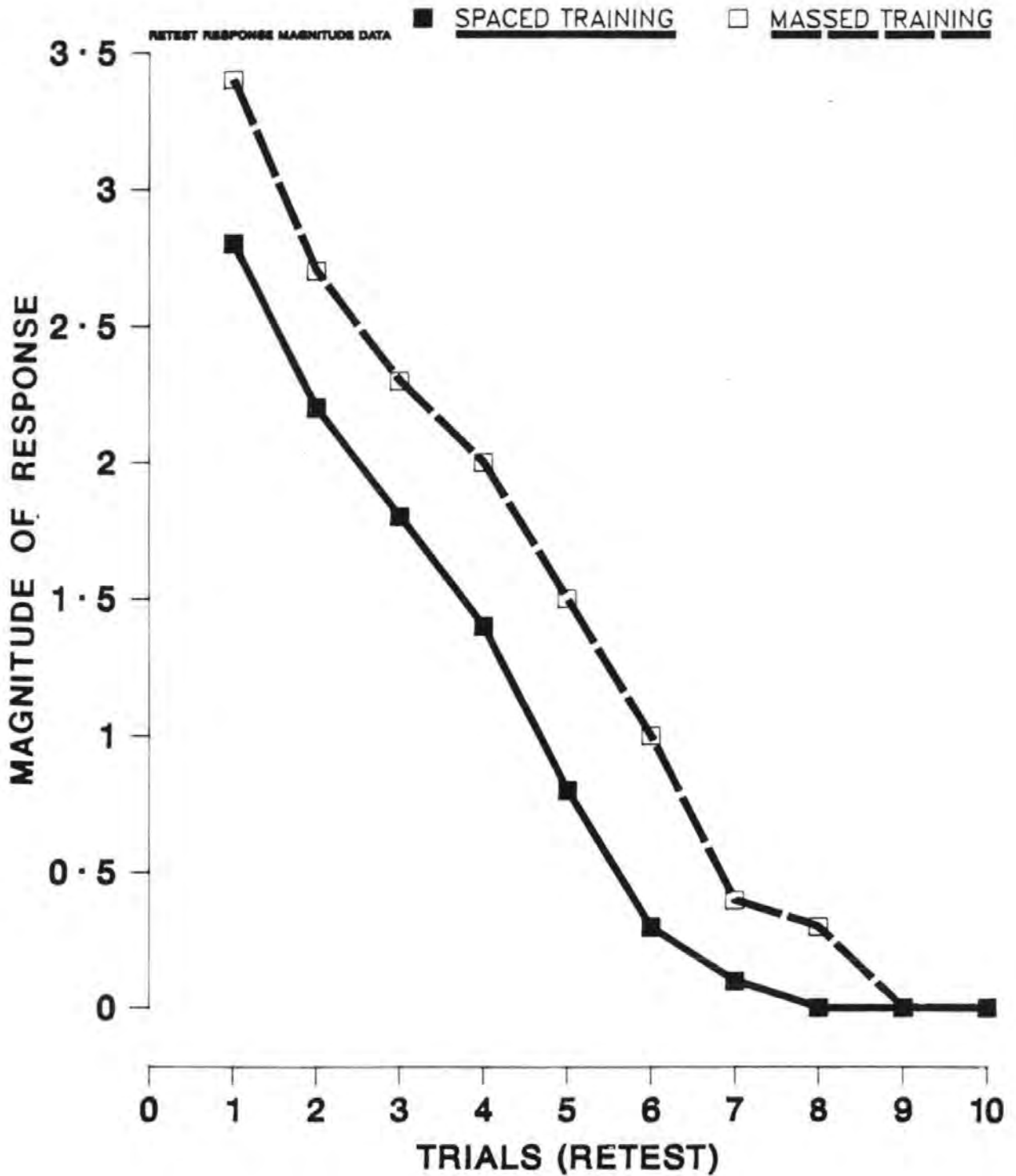


FIGURE 6.3b

EFFECTS OF MASSED AND SPACED TRAINING ON LONG TERM HABITUATION (24 HOURS)



the development of short term habituation at any of the 10 responses, and nor was there a difference between conditions in terms of mean response duration in Session 1 (all  $F'_s(1/18) = <1$ ). However, there was a difference between massed and spaced training in terms of response durations at retest ( $F(1/18) = 5.992, p<.025$ ) with snails in the spaced condition showing significantly smaller response durations at retest for responses 1 to 5 ( $F'_s(1/18) = 12.095, 32.385, 42.006, 27.210, p<.001$  and  $6.397, p<.025$ ). (See Figure 6.2).

Retention was evidenced from both spaced and massed training by significantly smaller responses in retest than responses evidenced in training ( $F'_s(1/18) = 12.31$  (spaced),  $p<.01$ , and  $5.11$  (massed),  $p<.025$ ).

A decrement with iterated stimulus presentations was evident in both Session 1 and retest for spaced training ( $F'_s(1/18) = 810.22$  and  $552.77, p<.001$  respectively) and in massed training ( $F'_s(1/18) = 885.93$  and  $742.598, p<.001$  respectively).

Analysis of response magnitude data supported the above findings. This analysis revealed a non significant effect of training condition ( $F(1/18) = <1$ ), but a significant effect of session ( $F(1/18) = 48.96, p<.001$ ), and trial ( $F(1/18) = 437.749, p<.001$ ) with significant interactions between training condition x session ( $F(1/18) = 7.918, p<.025$ ), session x trial ( $F(1/18) = 34.186, p<.001$ ). No other interactions reached significance ( $F(1/18) <1$ ). These results are illustrated in Figure 6.3a & b.

Analysis of simple main effects again revealed there to be no significant difference between massed and spaced

training in Session 1 ( $F(1/18) < 1$ ). Analysis of the development of habituation again revealed no difference between the two training conditions at any of the 10 trials ( $F(1/18) < 1$ ). In Session 1, there was no difference between groups in training response magnitudes ( $F(1/18) < 1$ ), but significantly smaller responses were evidenced in retest from the spaced training condition ( $F(1/18) = 6.100$ ,  $p < .025$ ). In the 24 hour retest retention was evident from spaced training but was absent in massed training.

### DISCUSSION

The results show that in short term habituation there was no difference between massed and spaced training. In long term habituation, 24 hrs post training, spaced training showed superior retention of habituation than did massed. With regard to the problems of measurement of habituation, it is interesting to note that with the less sensitive measure of response magnitude, no retention was evident in retest from massed training. This demonstrates the value of more than one measure of the learning. Further, it is interesting that, in short term habituation, there was no significant difference at any of the ten trials. One might predict a difference in trials either side of an interblock interval as, in spaced training, some recovery of the response should occur.

### EXPERIMENT 3

Another form of differentially distributed training uses larger interblock intervals by spacing training between sessions, and comparing performance with the same number of stimulus presentations massed into one session.

Carew et al. (1972) used this form of massing and spacing training. They reported individual response data for acquisition and retention of habituation from two, between session experiments in the marine mollusc *Aplysia*. In the spaced training condition, the *Aplysia* received 4 sessions of 10 stimulus presentations interspaced by 24 hrs. In the massed training experiment the same number of trials were included but all massed into one session. Their results show no difference in rate of short term habituation but superior retention at two retests intervals of 1 day and 1 week in the spaced training conditions. It was apparent that *Aplysia* in the spaced condition did show some differential development of habituation in that the initial response of each block of spaced training were of greater duration than their mass trained equivalents. This suggests that the response had shown some recovery in the inter block interval that was not apparent in the massed training animals. However, no detailed statistical interpretations were reported of these differences.

Experiment 3 serves to compare development of both short and long term habituation using multiple training sessions and analysing individual responses to specific stimuli presentations; in particular, the initial responses

in each block of spaced training.

#### METHOD

Subjects. Forty mature Helix aspersa were sampled from a population of 70 collected from the Bovisand Bay area of Plymouth.

Procedure. Laboratory housing, habituation procedure, stimuli and criterion for participation were as reported in Experiment 1. A multiple session, fixed number of trials per session paradigm was employed. Snails meeting criterion for participation were randomly allocated to a massed or spaced training condition. Six snails failed to meet participation criterion and were replaced by substitute animals from the same training condition.

Spaced training consisted of 3 blocks of 5 repeated presentations of the tactile stimulus with an ISI of 30 sec and an interblock interval of 6 hrs.

Massed training was generated by 1 block of 15 repeated presentations of the same tactile stimulus with an ISI of 30 sec.

Each training condition contained 20 snails, these were subdivided into to 2 retest conditions. One group from each training condition was tested for long term retention at 24 hrs post training and the other was tested 1 week post training. Retest interval was timed from termination of last response. Duration and magnitude of responses was recorded as reported in Experiment 2. Test apparatus was modified for this study in that it consisted of a 30 x 30 cm wooden board



with a 12 cm vertical surround to contain the snails during periods of no stimulation. Snails in both training conditions remained in the test apparatus in all rest periods during training, the interval to retest was spent in the home vivarium. Retests were conducted using 5 stimuli presentations with an ISI of 30 secs in both conditions.

## RESULTS

The results of Experiment 3 are depicted in Figure 6.4. Response durations were analysed in a 4-way mixed design ANOVA with between subjects factors of training (massed/space) and retest (24 hrs/1 week) and within subject factors of session (training Session 1, 2, 3 and retest). In massed training trial 1 to 5 equated to session 1, trial 6 to 10; Session 2 and trial 11 to 15). This revealed a significant effect of training condition ( $F(1/36) = 12.659$ ,  $p < .001$ ), session ( $F(1/36) = 932.592$ ,  $p < .001$ ), trial ( $F(1/36) = 1386.508$ ,  $p < .001$ ) and retest ( $F(1/36) = 5.856$ ,  $p < .025$ ) with significant interactions of training condition x session ( $F(1/36) = 237.734$ ,  $p < .001$ ), training condition x trial ( $F(1/36) = 52.811$ ,  $p < .001$ ), session x trial ( $F(1/36) = 228.005$ ,  $p < .001$ ), and significant 3 way interactions of training condition x session x trial ( $F(1/36) = 110.727$ ,  $p < .001$ ) and training condition x retest x session ( $F(1/36) = 8.180$ ,  $p < .01$ ). The 4 way interaction failed to reach significance ( $p < .5$ ).

Analysis of simple main effects revealed a saving in response durations across iterated sessions in spaced

FIGURE 6.4a

BETWEEN SESSION MASSING AND SPACING OF TRAINING  
TRAINING

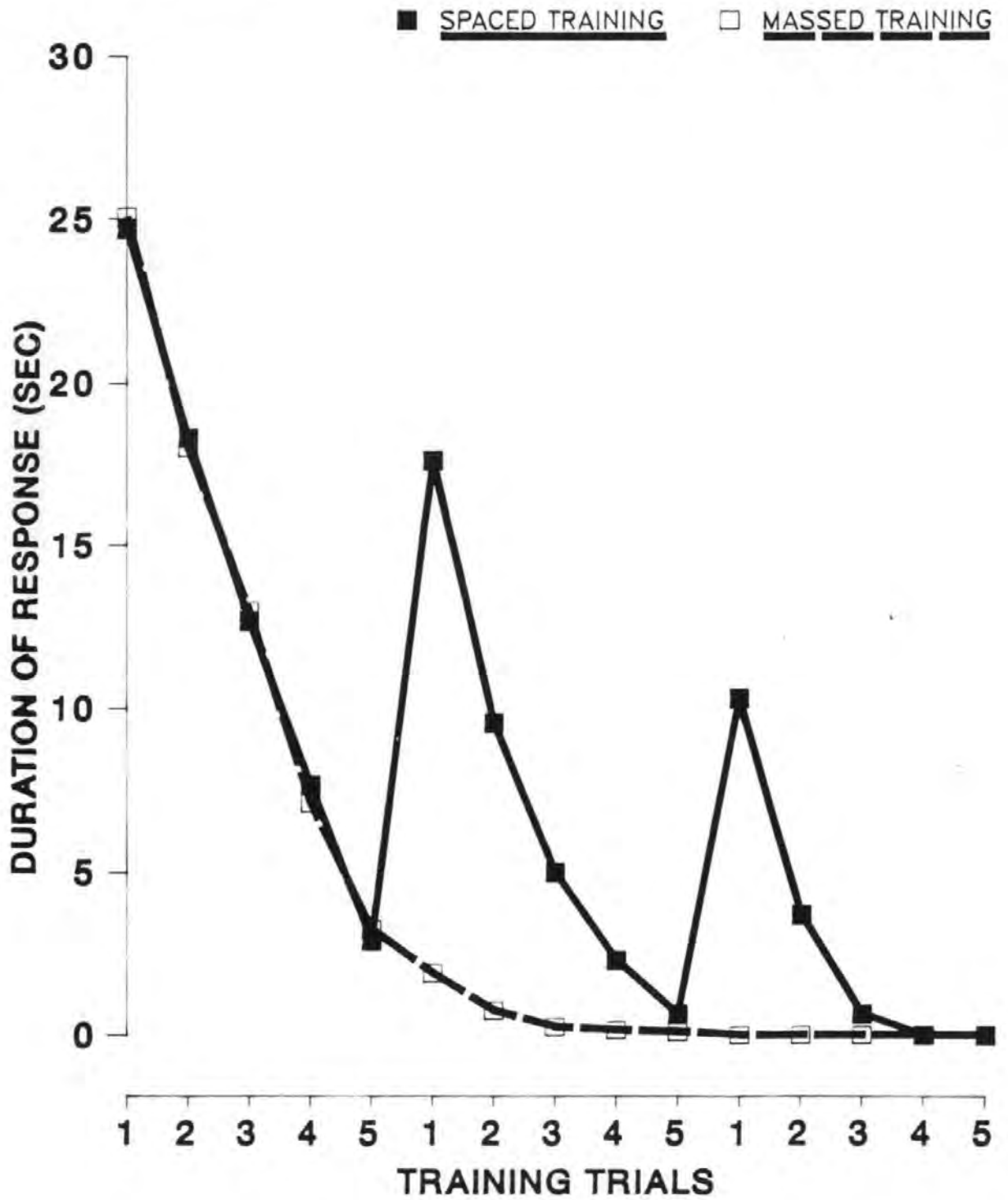


FIGURE 6.4b

RETEST 24 HR

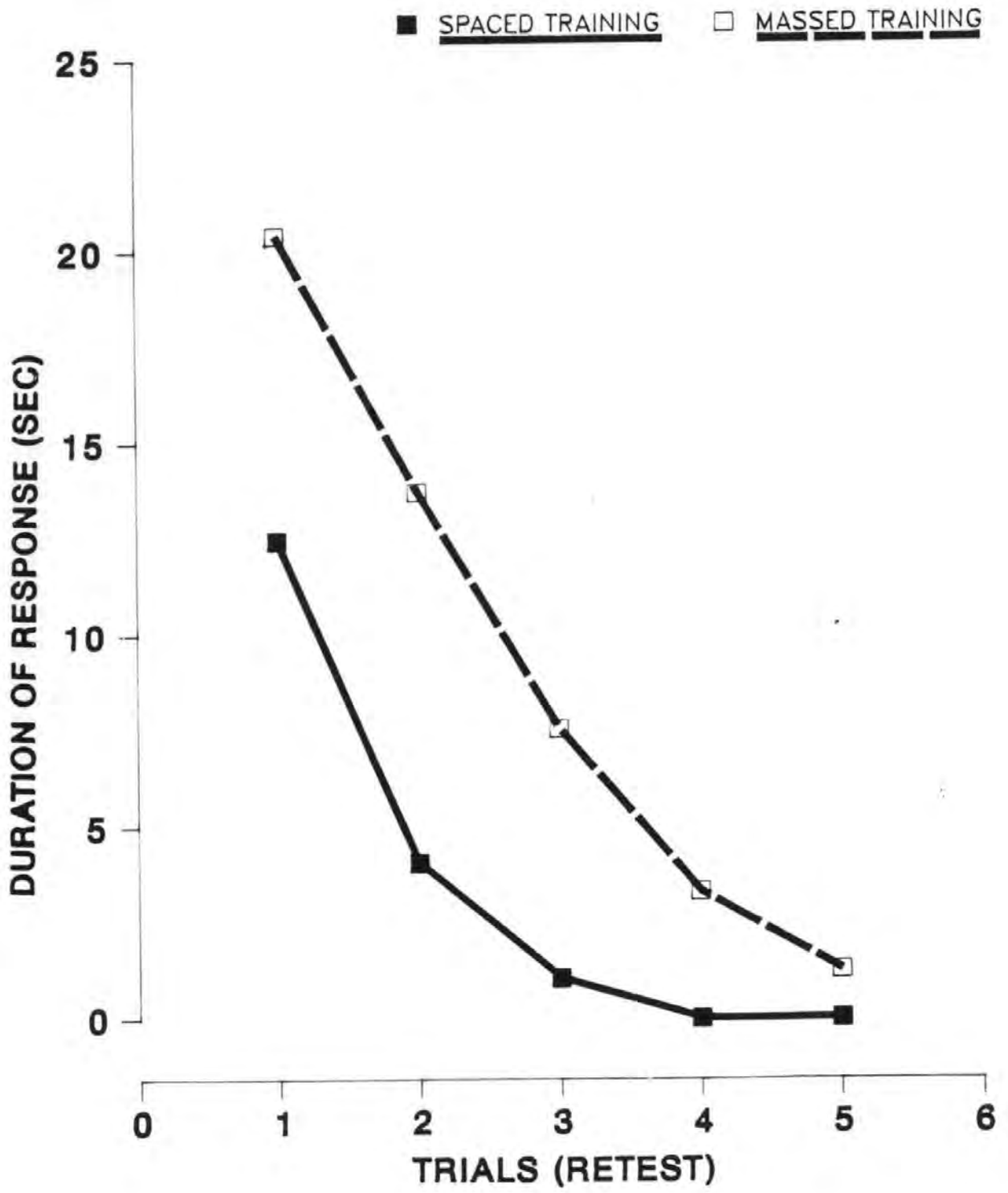
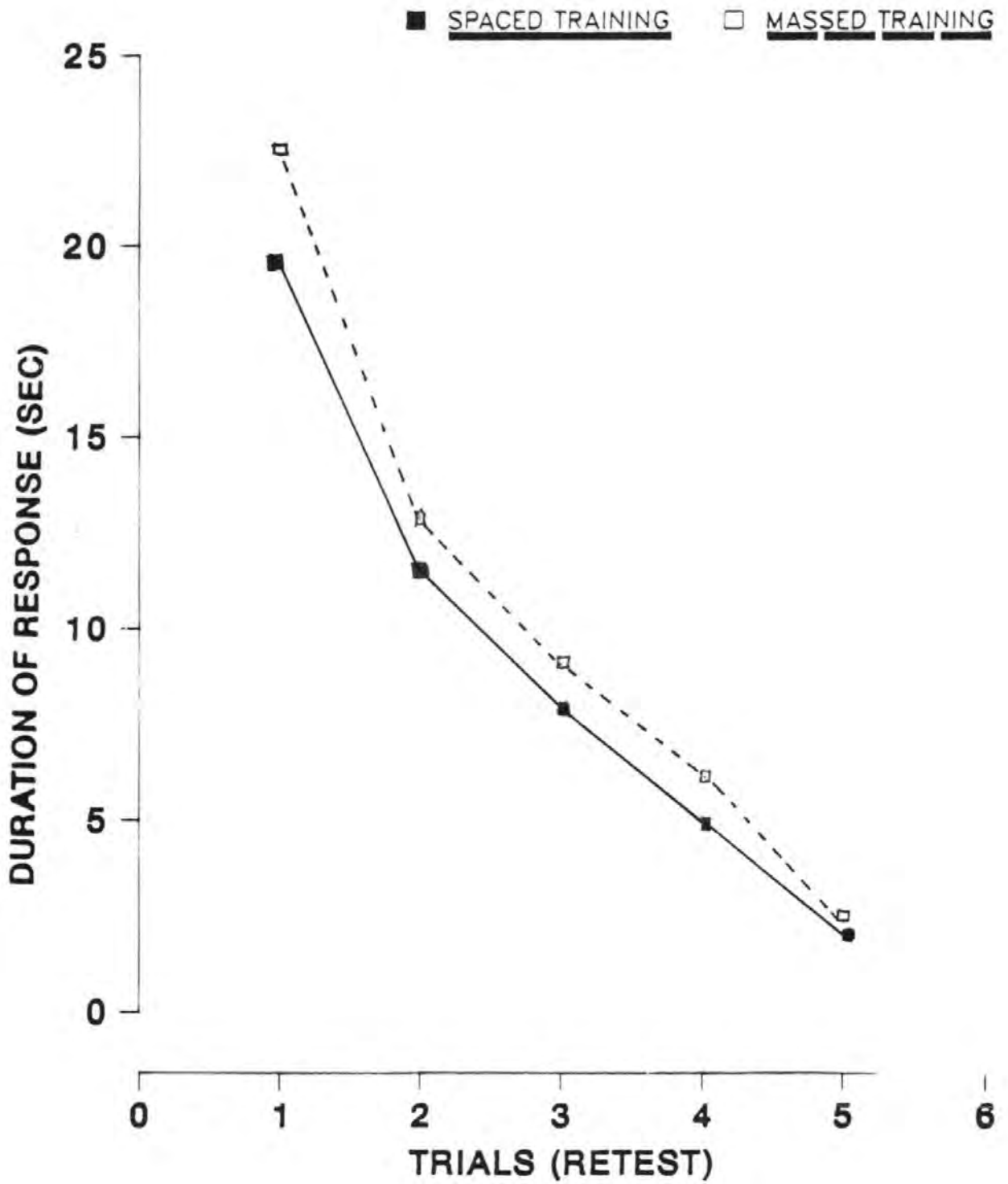


FIGURE 6.4c

RETEST 1 WEEK



training ( $F(1/36) = 120.72, p < .001$ ) and in massed trained snails ( $F(1/36) = 230.39, p < .001$ ). A comparison of massed and spaced training conditions at Training Session 1 revealed no difference in response durations between training conditions ( $F(1/36) < 1$ ), in Session 2, the spaced condition showed greater response durations than massed ( $F(1/36) = 24.48, p < .001$ ), this was also the case at Session 3 ( $F(1/36) = 5.31, p < .025$ ). At Retest 1, spaced trained snails showed smaller responses than massed ( $F(1/36) = 24.30, p < .001$ ) as was the case at retest 2 ( $F(1/36) = 26.97, p < .001$ ). The above results do suggest superior retention from spaced training, and, differential development of habituation across sessions. Further analysis compared each of the five response durations in each session between training conditions to further explore the above simple main effects. In the massed training group, Training Session 1 equates to trials 1 to 5, Session 2; 6 to 10, and Session 3; trials 11 to 15.

TRAINING SESSION 1. Across the 5 iterated presentations of the stimulus, there was a decrement of the response duration in massed ( $F(1/36) = 956.94, p < .001$ ) and spaced training ( $F(1/36) = 943.32, p < .001$ ). There was no difference in any of the 5 response durations between massed and spaced conditions (all  $F$ 's(1/36)  $< 1$ ).

TRAINING SESSION 2. The initial response in the spaced training condition was found to be significantly larger than the equivalent response in the massed condition ( $F(1/36) = 245.91, p < .001$ ). This shows recovery of the response in the interblock interval in the spaced condition. This is further

exemplified in that Responses 2, 3, and 4 were also larger in spaced training than in massed ( $F(1/36) = 77.26, 22.51, p < .001$ ; and  $4.61, p < .05$  respectively). There was no difference between conditions at response 5 ( $F(4/144) < 1$ ). Both groups were then close to asymptote.

TRAINING SESSION 3. Again the initial response was larger in spaced training ( $F(1/36) = 106.87, p < .001$ ). Response 2 in the spaced training was significantly higher ( $F(1/36) = 14.03, p < .001$ ) than that in the massed condition, but there was no difference responses 3, 4, and 5 ( $F(1/36) < 1$ ), all snails had reached asymptote (see Figure 6.4)

RETEST 1 (24 HOURS). The retest response durations were greater in massed training ( $F's(1/36) = 23.467, 19.04, 12.99, p < .001$ ; and  $7.61, p < .01$ , and  $5.02, p < .025$ ) showing greater retention than spaced. Both massed and spaced training conditions showed a decrement in response duration over the 5 iterate presentations ( $F's(1/36) = 920.765$  and  $90.308, p < .001$  respectively).

RETEST 2 (1 WEEK). Greatest retention was evidenced from spaced training with a smaller initial response evidenced in this condition ( $F(1/36) = 16.162, p < .001$ ). All the remaining 4 responses were again of shorter duration from spaced training than massed but this difference failed to reach significance ( $F's(1/36) = 1.359, 1.000, 0.639$  and  $0.275, p > .5$ ). Responses decreased over the five stimulus presentations in spaced ( $F(4/144) = 354.11, p < .001$ ) and massed training conditions ( $F(1/36) = 788.20, p < .001$ ).

## DISCUSSION

Both training conditions showed a decrement in response duration across repeated presentations of the tactile stimulus. Long term retention was evident 24 hrs and 1 week post training in both conditions, and spaced training resulted in greater retention at both retests.

In analysing the development of the habituation across training sessions, it is of interest that the initial response of each block of spaced training was larger than the same response in the massed training group. This shows some recovery of the response in the interblock interval which was absent in massed training. It has been suggested (Kandel, 1976) that it is this difference which is responsible for greater long term habituation evident in the spaced training condition.

## GENERAL DISCUSSION

All forms of massed and spaced training employed resulted in some long term habituation. In both of the within session manipulations of training (Experiments 1 & 2) and the between session study (Experiment 3), spaced training resulted in more retention of the habituation than did the massed training condition.

The main difference between the effects of the two forms of massed and spaced training used (within and between session) is that, in single session manipulations of training, there was no differential development of

habituation despite the predicted long term effect. Conversely, with spaced between session training there was evidence of differential development of the habituation in that responses were longer at the beginning of each block of training compared to equivalent trials in massed training (Trials 1, 6 and 11).

Kandel (1976) and Castellucci and Kandel (1976) have suggested such a re-evocation of the response at the beginning of each block of trials to be responsible for spaced training's superiority in producing long term habituation. They have argued that between each block of trials, there would be an opportunity for recovery and reinstatement of the calcium current, mobilizing transmitter release from the storage pool and binding vesicles to release sites. Thus, the response could be re-evoked at the beginning of each block of trials, but the repeated transfer of transmitter release sites may produce a profound depletion of the storage pool. Further, such a depletion might give rise to the inhibition of transmitter synthesis, or prevent the transport of additional transmitter substances to the storage pool from the cell body. Alternatively, profound depletion of the storage pool might prevent further transfer from this pool until it has been restored to a given level, and this may be slow in real time. In massed training, the interval between stimuli is much shorter with consequently less recovery and reinstatement of calcium current taking place. It would follow in this circumstance, that the short term effects of stimulation would not be passed to the storage pool as there



would be less opportunity for the mobilization of transmitter from the storage pool to the release sites. From this, Kandel suggests, as the storage pool would not suffer profound depletion, long term retention would be present but limited.

Kandel's interpretation of the physiological basis of the effects is based on the empirical evidence of Carew et al. (1972) who used between session designs in the spacing of training in *Aplysia* (see Chapter 2). The importance of the initial response level of each block of spaced training was evident in *Aplysia*. However, the results of Experiment 1 and 2 do still show superior retention from spaced training without the observed greater recovery between blocks. On the other hand, in all training conditions there will be an interval between stimuli presentations, the physiological processes are not all or none, They are quantifiable entities that may still occur without any effect on overt measurable behaviour. Thus, the effects predicted could occur in the way predicted without the observable differential development of the short term habituation.

Recently, Kandel and co-workers have addressed the actual time course of acquisition and retention of learning in physiological terms (Goelet, Castellucci, Schacter, & Kandel 1986). They suggest that a single learning event initiates several memory processes with different time courses of retention. Short term memory is said to involve covalent modifications of pre-existing proteins, whereas they suggest that long term memory requires the expression,

during learning, of additional genes (see Chapter 1). The expression of genes in this model requires the the secondary messenger c-AMP initiated by neurotransmitter release. Although the inter block interval of spaced training would allow greater release, the smaller interstimulus intervals of massed training would also produce some c-AMP during training. Consequently, spaced training would result in superior retention, but these sub-cellular events would be subject to inductive changes in transmitter availability and release and could occur in the smaller inter block intervals of the within session experiments reported above.

The interval between the blocks of training in the spaced condition of Experiment 2 may have been too short to allow enough recovery of the response prior to the next stimulus presentation. However, it could still generate differential development of habituation between the massed and spaced training conditions at a physiological level such as that suggested by Kandel. This would result in the superiority of spaced training in terms of long term habituation which has been reported in all three experiments and, in particular, in the two experiments in which multiple measures of habituation failed to detect differential development of short term habituation at the level of gross behaviour.

Kandel's theory suggests a differential process view of short and long term habituation and thus supports the research reviewed in Chapter 2. The eight experiments reported in Chapters 5 and 6 provide empirical evidence to support such a view.

Investigations of massed and spaced training effects and ISI effects on short and long term habituation are included and of consequence to this thesis for two reasons. First, they support the growing behavioural literature reviewed in Chapter 2 which suggests differential process views of these two processes. Second a similar conclusion has also been derived from the limited studies of protein synthesis involvement in short and long term habituation (see Chapter 1, section 1.2). Further, the experiments reported here demonstrate that habituation of the dorsal antennae withdrawal response in the snail shows similar parametric characteristics as other vertebrate and invertebrate examples of habituation.

Having established short and long term habituation of the dorsal antennae withdrawal response in the snail Helix aspersa, and explored the parametric characteristics of this form of learning which indicate a difference in underlying processes, A comprehensive investigation of the effects of various protein synthesis inhibitory drugs on this learning can now be completed. This would establish a role of protein synthesis in habituation and further clarify the differential process debate.

## CHAPTER 7

### PROTEIN SYNTHESIS INHIBITORS, SNAILS AND INJECTION METHODOLOGY

Research in the initial chapters of this thesis established short and long term habituation of the dorsal antennae withdrawal response in the snail *Helix aspersa* (Chapters 3 and 4) and provided behavioural evidence in support of a multiple process view of these two phenomena (Chapters 5 and 6).

The remaining chapters of this thesis investigate the behavioural effects of injections of several doses of four PSI's, puromycin, actinomycin D, anisomycin and cycloheximide. These drugs are known to be taken up by neuronal tissue in *Helix aspersa*, similarly these authors report that these antibiotics disrupt protein synthesis almost immediately post injection and up to 2 hours post training at doses ranging from 5 $\mu$ g to 500 $\mu$ g (Kerkut, Emson, & Walker, 1973; Kerkut, Oliver, Rick, & Walker, 1970; Sokolov, 1974. Further, Schwartz, Castellucci and Kandel (1971) reported that these drugs also disrupt protein synthesis in the tissues of the marine snail *Aplysia californica*. While there have been biochemical investigations of uptake and inhibition of these drugs in the snail, investigations of their behavioural effects have been limited.

Research reported in this chapter develops a suitable injection methodology, and cites evidence of antibiotic

uptake, and protein synthesis inhibition. The time course of such action is shown. In Chapter 8, the development of a battery of behavioural tests to screen these drugs for behavioural side-effects is reported. This initial programme of work is then completed with an examination of the dose-effect relationship of the drugs at safe

doses established in Chapter 8.

The second programme of work establishes the main effects of inhibiting protein synthesis on short and long term habituation and addresses alternative explanations of PSI work such as state dependency effects, and short and long term latency effects (See Chapter 10 and 11). Chapter 12 completes this programme by investigating the effects of differential levels of stressing of the animals and the PSI effects.

The third programme investigates the time of effect relationship of PSI effects on short and long term habituation and discusses the implications of the results in terms of a depletion of constitutive proteins or induced gene expression (See Chapter 13).

The final programme investigates the differential effects of PSIs on short and long term habituation with a demonstration that short term habituation shows the normal parametric characteristics established in Chapters 3 to 6 in the presence of PSIs (see Chapters 14 and 15) and that the amnesia produced by the PSIs in long term habituation is complete (see Chapter 16). This programme is concluded by a series of experiments which suggest gene expression as a mechanism of long term habituation.

The initial phase of study reported in this chapter consisted of 'underpinning research' which investigated the uptake of PSIs into neuronal tissue from injection.

## 7.1 THE DRUGS

The protein synthesis inhibitors that have been most frequently used in studies of learning and memory are Anisomycin, Actinomycin D, Puromycin, Acetoxycycloheximide and Cycloheximide. Four of these antibiotics were investigated here. These antibiotics (anisomycin, actinomycin D, puromycin and cycloheximide) represent three different classes of drugs, and each class blocks protein synthesis at a different stages of metabolism and by different mechanisms of action (see Chapter 1).

In a typical protein synthesis inhibition study, an animal is trained shortly after drug injection and retention is tested at given intervals post-training.

## 7.2 INJECTION TECHNIQUES AND SITE OF ACTION

Initially, the injection methodology most commonly employed in PSI research was initially intracerebral injection of the antibiotics. A more contemporary approach has been the employment of peripheral administration to circumvent possible complications of intracerebral injection procedure such as brain damage and electrophysiological disturbance (Bohdanecka et al., 1967; Dunn, 1975), although, some authors have argued that the antibiotics injected

peripherally also act peripherally.

This seems unlikely if we compare the relative doses required to cause amnesia from peripheral injections with amnesic doses from cerebral injection (see Barondes, 1970). Some tangential support for this conclusion is provided by Dunn et al. (1977) who showed that peripheral administration of pactamycin and emetine, which inhibit protein synthesis in the liver and adrenals but not in the brain, are not amnesic. The authors suggest this is possibly because the drugs cannot cross the blood-brain barrier.

Additional evidence against peripheral actions of the antibiotics is the regional specificity of their action. Eichenbaum et al. (1976) found that cycloheximide was amnesic when locally injected into the striatum, hippocampus, amygdala, and posterior lateral thalamus but not the anterior lateral thalamus, midbrain reticular formation, or posterior or anterior medial cortex. Inhibition of cerebral protein synthesis in these regions was verified by autoradiography. Partlow et al. (1978) found cycloheximide amnesic in the amygdala but not in the internal capsule, and Boast and Agranoff (1978) found streptovitacin A amnesic in the hippocampus but not in surrounding cortical regions or the internal capsule. The above evidence is further reviewed by Dunn (1980). The evidence suggests that antibiotics have a central rather than peripherally mediated amnesic effect, and this supports the peripheral injection and central effect hypothesis.

With invertebrates, peripherally administered drugs do have a central effect on neuronal tissue (e.g. Kerkut et

al., 1971). All injection protocols employed in my laboratory involve injection into the foot section beneath the head of the snail from a Gillette surgical syringe with a needle penetration of approximately 5mm. This penetration and location of injection ensured maximal infusion of drug in the area of the subserving ganglion. From pilot studies (Ray, 1984), it was found that the most controllable method of injection was to administer it while the animal was still inside its shell. In this enclosed form, the opercula of the shell contains the foot section immediately dorsal to the ganglion. This, in turn, is covered by a thin membranous sheath. The snail was held inverted by one hand under a fixed magnifying glass (Magnification was found to facilitate more accurate control of depth of penetration and standardise injection procedure), and injection was administered by the other hand. It was found that this injection technique was the most suitable and served to encourage snails to emerge from their shells after an initial copious secretion of mucus.

### 7.3 DOSES OF DRUGS EMPLOYED

A review of the literature revealed a wide variation of doses employed (see Chapter 1). As a consequence of this, the following doses of the four antibiotics were tested in the initial three series of studies (see Chapters 8, 9, 10) to find the most suitable doses.



## DOSES OF DRUGS EMPLOYED

0.00025mg/ml.....	0.1ml injection.....	0.025yg/snail
0.0025mg/ml .....	0.1ml injection.....	0.25yg/snail
0.025mg/ml.....	0.1ml injection.....	2.5yg/snail
0.25mg/ml.....	0.1ml injection.....	25yg/snail
2.5mg/ml.....	0.1ml injection.....	250yg/snail

\* doses are given as mg per ml with this dose converted to yg per snail from the standard volume injection used; 0.1 ml. Snails of approximately the same weight were used in all drug studies to avoid the problems of having to use dose to weight ratios which could lead to animals in the same treatment group receiving different doses. The availability of snails facilitated this method in that collection of samples could be specific on a criterion of animal weight. N.B. The symbol y is used to represent MICRO ( $\Upsilon$ ) throughout this thesis.

### 7.4 PURITY OF ANTIBIOTICS

All antibiotics were supplied from SIGMA Chemical Co. with tested and guaranteed purity of the drugs. The drugs were placed in solution with Physiological saline to the above specified doses, and pH was maintained in all solutions to pH 6.5. All solutions were made up by the Department of Chemistry, Plymouth Polytechnic.

### 7.5 UPTAKE OF DRUG AND INHIBITION OF PROTEIN

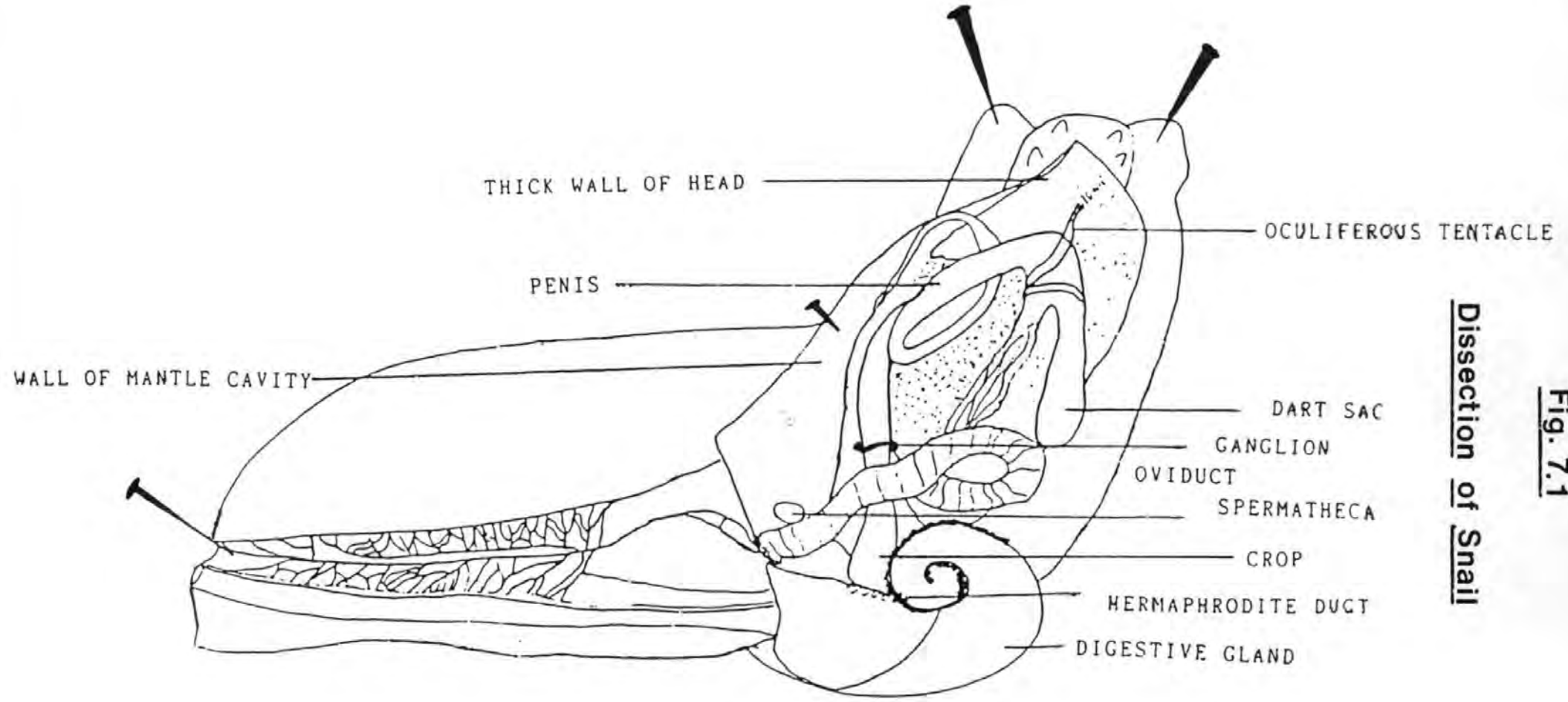
## SYNTHESIS IN NEURONAL TISSUE

It is well established that antibiotics do cross the blood brain barrier of vertebrates (Reviewed Davis and Squires, 1984). Similarly, labelling studies have demonstrated the parameters of uptake in molluscan nervous tissue (Schwartz, Castellucci, & Kandel, 1971), and more specifically in Helix aspersa uptake of cycloheximide and actinomycin D into neuronal tissue has been demonstrated by radiolabelling (Kerkut et al., 1971). Radioactive labelling studies also reveal uptake of Anisomycin (Sokolov, 1974).

A series of Leucine-3H incorporation studies were conducted in support of the behavioural aspects of this thesis by the South Yorkshire Area Health Authority (Unpublished data) to establish that these drugs at the above doses inhibited protein synthesis in neuronal tissue of the snail after pedal injection. Facilities and expertise for this work were not available in this department. As this research does not constitute part of the thesis, it is only summarised below as it relates to the behavioural research reported in the thesis. (See Appendix 7.1)

### METHOD

Each snail received a simultaneous ganglionic injection of the label and a pedal injection of PSI. After a prescribed incubation period the snail was killed and the ganglia removed (see Figure 7.1). Twelve incubation times were investigated from immediately after



Dissection of Snail

Fig. 7.1

injection then every 15 min up to 165 minutes post injection. This study was conducted for all four antibiotics. Each study involved 30 snails in 6 equal groups. A saline injected group, and five PSI injected groups, one group for each of the 5 doses outlined above.

The time course of incorporation of leucine- $^3\text{H}$  (380 counts/minute per pmole) was then compared between snails that had received drug at a given dose to saline injected snails.

## RESULTS

### Anisomycin

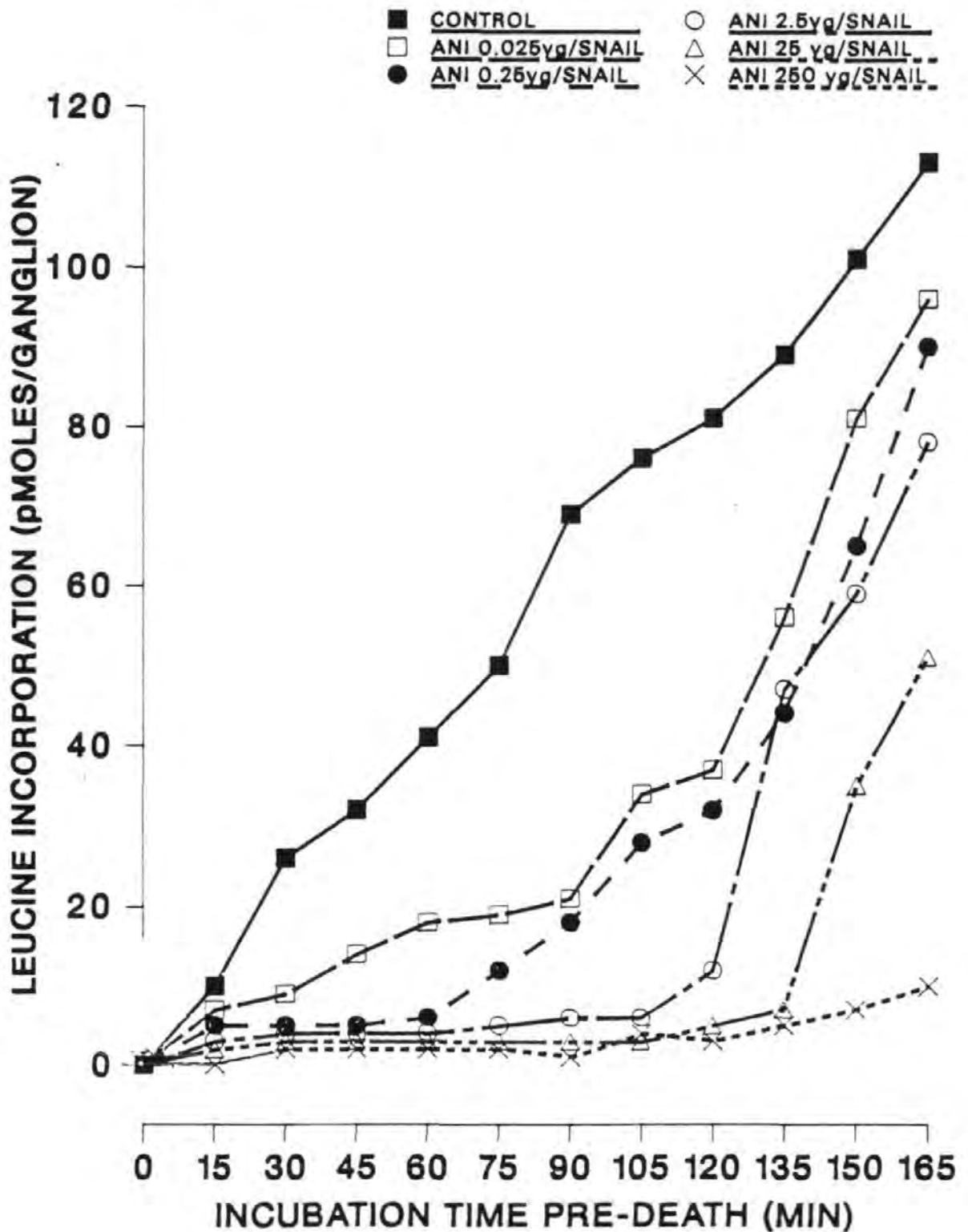
Anisomycin was found to inhibit protein synthesis at all doses (see Figure 7.2). The onset of inhibition in the ganglion appeared to be immediate from peripheral injection and the extent of inhibition varied as a function of dose. The inhibitory effect of the drug at all doses up to dose 4 had terminated by 2 hrs 15 mins post injection. Dose 5 still appeared to inhibit protein synthesis beyond the maximum time sampled.

### Puromycin

The results for puromycin showed virtually identical uptake and inhibition characteristics as anisomycin in that the drug inhibited ganglionic protein synthesis at all doses, and extent of inhibition was proportional to dose.

FIGURE 7.2

EFFECTS OF ANISOMYCIN ON LEUCINE INCORPORATION IN INTACT SNAIL



Dose 5 was again found to be inhibiting protein synthesis at the maximum time sampled. All the lower doses inhibited protein synthesis up to 2 hrs. Lesser doses than Dose 4, however, showed a lesser magnitude of protein synthesis inhibition (see Figure 7.3).

#### Actinomycin D

Although the similarity of parameters of inhibition for anisomycin and puromycin could have been predicted because of their similar modes of action, such a prediction does not necessarily follow for actinomycin D, as it has a different mode of action. However, it did show similar uptake and inhibition characteristics and dose effects (see Figure 7.4).

#### Cycloheximide

Cycloheximide was found to be taken up almost immediately. Like the other three antibiotics tested, cycloheximide did not inhibit protein synthesis at Dose 1, but all other doses did inhibit protein synthesis. Again, this effect was found to vary with dose. Inhibition lasted for approximately 2 hrs for Dose 2 to 4. Dose 5 was still inhibiting protein synthesis at the maximum time interval sampled. These results are illustrated in Figure 7.5.

FIGURE 7.3

EFFECTS OF PUROMYCIN ON LEUCINE INCORPORATION IN INTACT SNAIL

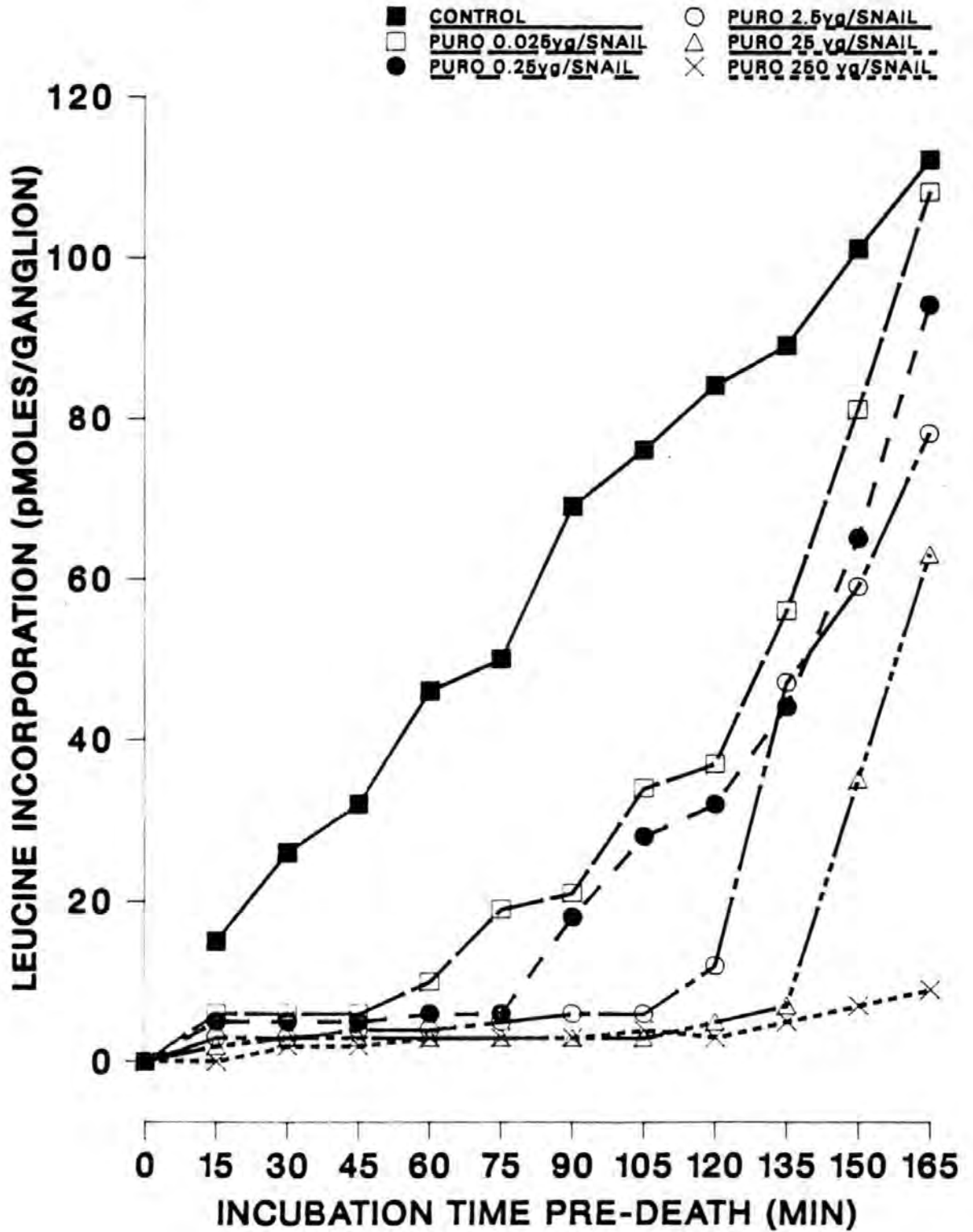


FIGURE 7.4

EFFECTS OF ACTINOMYCIN D ON LEUCINE INCORPORATION IN INTACT SNAIL

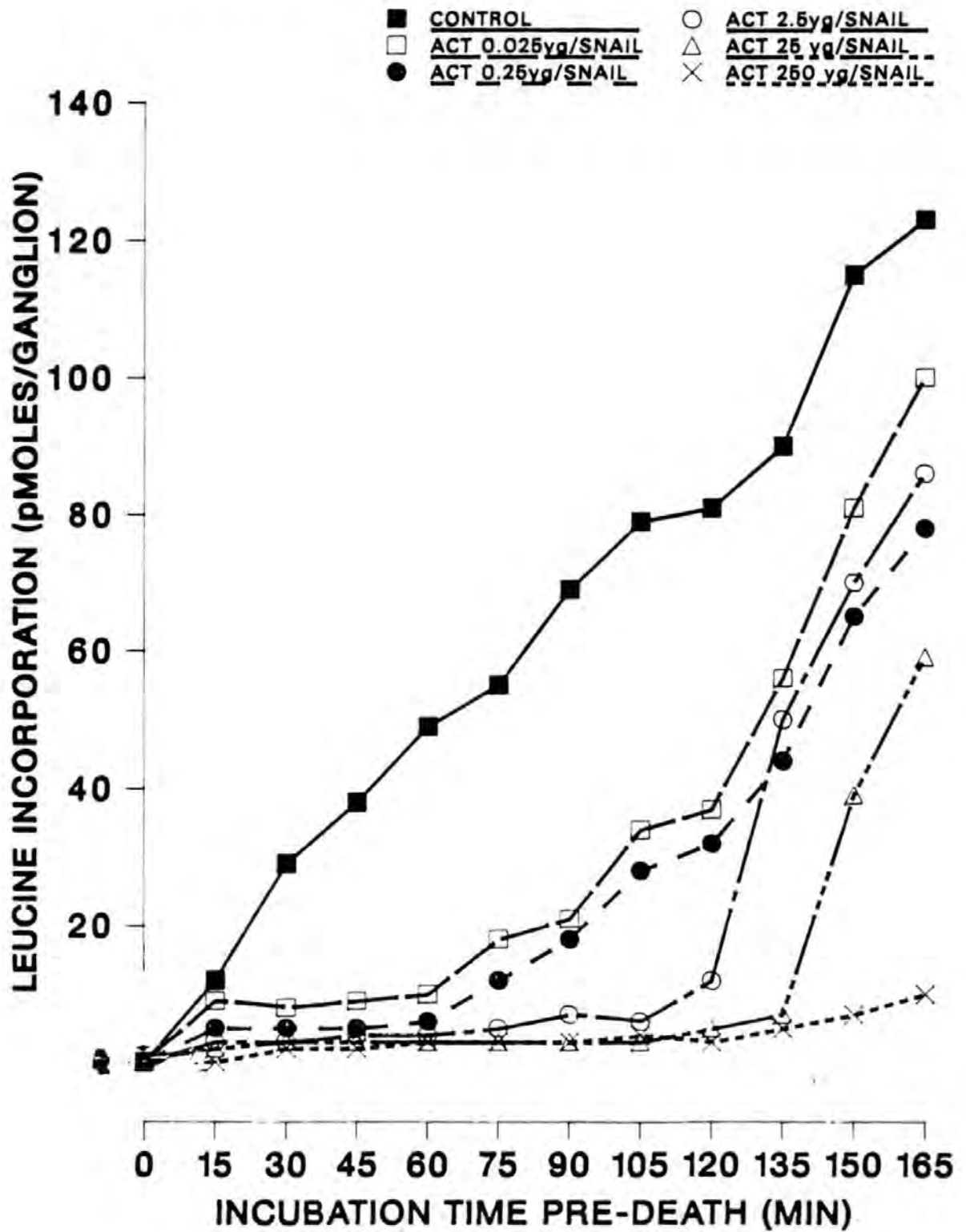
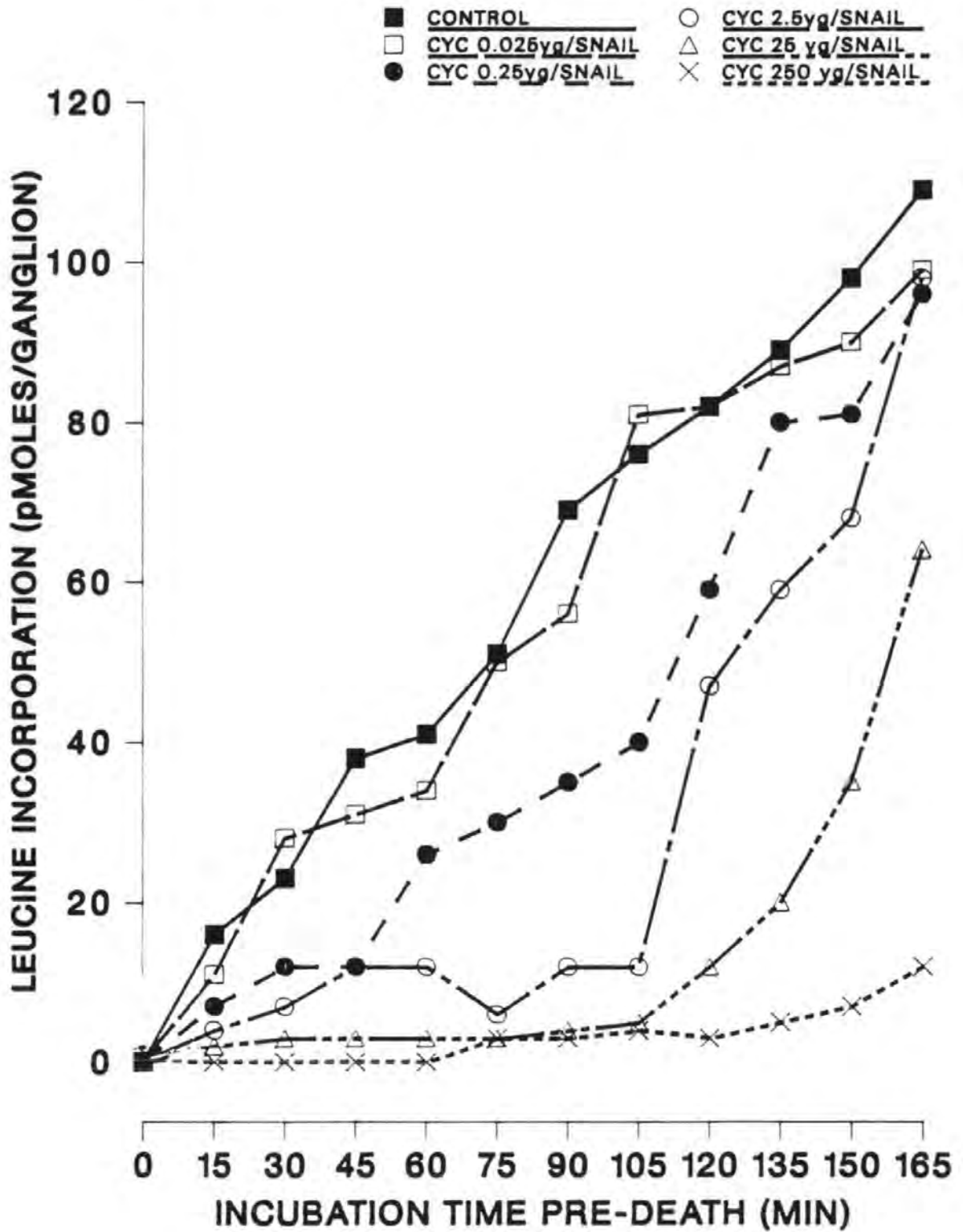




FIGURE 7.5

EFFECTS OF CYCLOHEXIMIDE ON LEUCINE INCORPORATION IN INTACT SNAIL



## DISCUSSION

These preliminary studies, conducted in support of the behavioural aspects of the thesis, suggest that from a pedal injection of antibiotic, the drugs uptake into neuronal tissue and immediately disrupt protein synthesis. The extent of inhibition was dependent upon dose, and the four antibiotics appeared similar in the parameters of inhibition. A general conclusion is that all drugs at doses up to dose 4 inhibit protein synthesis in the ganglion reversibly for approximately 2 hrs. The time of effect of these drugs relative to injection is explored behaviourally by a comparison of injection time relative to training and observing the effects of the drugs on short and long term habituation (see Chapter 13). Before this can be done, however, the drugs must be investigated for any side effects which could debilitate the snails. This investigation is carried out in the next chapter.

THE DEVELOPMENT OF A BATTERY OF BEHAVIOURAL TESTS TO SCREEN  
ANTIBIOTICS FOR GENERAL EFFECTS ON SNAIL BEHAVIOUR

It is unequivocally established that anisomycin, puromycin, actinomycin D, and cycloheximide all reversibly inhibit protein synthesis in living tissue (Davis and Squires, 1984) and the snail is no exception (Kerkut et al., 1973; see Chapter 7). However, little research has been conducted on the effects these drugs may have on general behaviour as well as any specific effects on learning.

Throughout the literature, many authors have remarked upon the possibility that the supposed amnesic effect of the antibiotics could be attributed to general debilitation of the animal by the drugs. Reports of induced sickness are common (Barondes & Cohen, 1968; Davis et al., 1980; L.B. Flexner et al., 1966; Squire & Barondes, 1974; Squire, Geller & Jarvick, 1970). Other authors have attributed the amnesic effect to abnormal locomotor activity (J.B Flexner et al., 1962; Gutwein, Quartermain, & McEwen, 1974; Segal et al., 1971; Squire & Barondes, 1974; Squires et al., 1970). A further suggestion, primarily pertaining to puromycin, is that the reported memory loss is due to abnormal electrical activity (Davis & Squire, 1984), inhibition of adrenal sterogenesis (Garren, Davis, Crocco & Ney, 1966; Garren, Ney & Davis, 1965; Nakajima, 1975, 1976) or abnormal catecholamine biosynthesis (Bloom et al., 1977; Cooper et al., 1978; L.B. Flexner, & Goodman, 1975; Flood et al.,

1984; Goodman, Flexner, & Flexner, 1975). Investigation of all these 'side effects' has been scant, and limited to the laboratory rat, and with no attention paid to dose of drug. The issues of side effects and alternative explanations of results are more fully discussed in Chapter 1.

Before any further progress can be made in this field, side effects of these drugs must be investigated more thoroughly. Consequently, in this chapter, a series of studies were conducted to develop a suitable test battery for application in the snail to screen candidate antibiotics for potential side effects.

The concept of behavioural test batteries to screen drugs for side effects has its origin and development in the area of psychopharmacology and teratology. In drug testing and development, new pharmacological products are screened on a battery of behavioural tests developed to show effects on a variety of aspects of normal behaviour. Such test batteries can be of general use or can be designed with specific drug groups in mind.

The issue of side effects in research on pharmacological disruption of memory has primarily been addressed by comparing the effects of different drugs with different modes of action under the premise that these drugs should have different side effects. The logic of such an approach is questionable, and contamination of results with side effects remains a recurrent criticism of the area.

In this chapter, a battery of behavioural tests is reported which screen antibiotics at a variety of doses for possible disruption of normal snail behaviour before these

drugs are investigated for their effects on short and long term habituation. In the development of any test battery, the normal baseline behaviours of the target animal must be represented. Further, the method of introducing the drugs into the animal's system must be screened for effects on general behaviour. For the snail *Helix aspersa*, the following tests were included:

- 1) Open Field Activity
- 2) Defensive withdrawal
- 3) Activity levels
- 4) Test of olfactory abilities
- 5) Feeding behaviour
- 6) Righting reflex

This battery of tests was developed as a hierarchical method of screening which initially screened for motor deficits and then if the drug had no such effects, tested sensory and coordination effects. Tests 1, 2 and 3 were all designed to investigate the effects of antibiotics on motor abilities. If a given dosage of the drugs had an effect, that dosage was removed from further testing. Drugs that showed no effect on these tests were then screened on the final 3 tests.

## 8.1 OPEN FIELD TEST

### Aim

Field observations of snail behaviour have shown the predominant behaviour of snails to be exploration or foraging (Ray, 1986b). Therefore, it is important to determine the effects these drugs may have on this class of behaviour, so an 'Open Field' test was developed for the snail. This is a standard test used in behavioural pharmacology for screening drugs for locomotor defects in rats.

### Design

Snails were placed in a black perspex box measuring 1 x 1 m with a vertical surround 30 cm high. The surround was lightly coated with odourless oil (Mazola) to prevent the snails climbing the sides of the apparatus. Each animal was placed in a central, etched circle, 2 cm in diameter at the beginning of each trial. The snails movement was then recorded for 15 min. Movement was recorded by covering the box with a transparent rigid acetate sheet which was subdivided into 2 cm squares. The snails ambulations were observed from above the apparatus and traced onto the acetate using a dry marker pen. Upon the completion of the trial, the acetate was removed, and the number of boxes the snail had entered was calculated. The snail was then removed, marked, and placed in a holding vivarium for a long term mortality study. The apparatus was thoroughly washed with hot water between trials to avoid any possible

confounds due to slime or smell remaining in the apparatus from the previous animal. All testing was conducted between 2400 hrs and 0630 hrs when the animals should be at their most active. Lighting during this test and all others was provided by a 60w red bulb in an angle lamp positioned 1 m above the floor of the apparatus.

## 8.2. WITHDRAWAL REFLEX

### Aim

The major defence mechanism of the snail is its ability to withdraw its antennae into its head and, in extreme danger, to withdraw completely into its shell. As habituation of this reflex is the learning behaviour investigated in this thesis, any debilitating effects of antibiotics on the snail's ability to withdraw to aversive stimuli is of major consequence to interpretations of the final experiments.

### DESIGN

This test was sub-divided into two component tests. An initial test consisted of snails being lifted onto a laboratory dissecting board where they remained unmolested for one minute. Each snail then received a tactile stimulus to the head approximately 2 mm caudal to the dorsal antennae. The stimulus used was an 80-mm length of solid aluminium rod with a rounded end and a diameter of 3mm. The duration of the response elicited was recorded using a stop watch (Smiths 1/10th).

In the second test each snail was held by its shell until fully extended from their shells approximately 5cm

above the base of a waxed laboratory dish. It was then gently lowered head first until the antennae touched the waxed surface. The response elicited was measured again in terms of duration. Each snail was screened on both tests.

### 8.3. ACTIVITY LEVEL

#### AIM

This test screens for any antibiotic effect on the snail's general level of activity. It has been suggested that antibiotics do affect activity levels in the rat (Segal and Barondes, 1971; Squires et al., 1970). If the level of general activity of the snail is affected by the drugs, this could affect their performance on learning tasks.

#### DESIGN

A test of general activity levels was designed such that a large group of animals could be injected with a given dose of a drug, and a similar number could be given the same dose of other drugs, saline, or receive no injection. All animals can be screened together on this test. After injection, the snail was numbered with paint on the shell which was colour coded for drug condition for ease of identification. The snail was then rehoused in an electric plant propagator (Woolworths) for 12 hours. This was illuminated by a 60w red bulb. The propagator remained on throughout the experiment to maintain a constant humidity and temperature that would encourage snail activity.

Throughout the 12 hrs, the snails were observed every hour, and the number of snails in each condition which had their heads extended from the shell and showed either head



movement or ambulatory behaviour was recorded for that observation. All snails that were dormant (i.e. remained in their shells at a particular observation) were recorded as inactive.

#### 8.4. DETECTION OF A HIDDEN FOOD SOURCE IN OPEN FIELD

##### AIM

This test provides a means of evaluating effect of antibiotic drugs on the olfactory capabilities of the snail. Olfaction is the snails' most important and well developed sense. If this sense is impaired, it could affect the snails' general behaviour or perception of incoming stimuli and confound any drug effects on learning.

##### DESIGN

A modified open field apparatus was used for this test. Within the apparatus were two white opaque perspex 15 cm cube boxes with clear top sections. Each had a 2 cm hole at floor level facing the snail start point which was an etched 2 cm circle at the opposite end of the apparatus. The food source was 75 ml of pineapple juice contained in a petri dish that was painted white. Permeation of the odour was facilitated by a series of 12, 3-mm holes that were drilled around the top of the box. At the commencement of a trial, the test snail was placed in the start circle, and, when the snails shell was clear of the circle, a stop watch was started. The trial was complete when the food source had been reached and the snail reared to the food. If a snail had not located the food within 5 minutes, the trial was deemed to have finished. The time taken to reach the food

was recorded.

## 8.5. FEEDING BEHAVIOUR

### AIM

A decrease in the ability of the snails to feed on presented food would be an indication of a general debilitating effect of the drugs. Such a general effect would confound any conclusion drawn concerning drug effects on learning.

### DESIGN

A population of snails that had been food deprived for 1 week were sampled in the test. Each snail was placed in a white laboratory dish (30 x 30 cm). A 1 gm lettuce leaf was presented directly in front of and in contact with snails head. The snail remained with the food source in subdued lighting for 20 minutes. After this period the remaining food was removed from the apparatus and weighed, and the weight consumed was calculated.

## 8.6 RIGHTING REFLEX

### AIM

One of the most complex behaviours that snails show naturally is their ability to right themselves when they are placed on the backs of their shells. This behaviour necessitates the coordination of both motor and sensory abilities and has been observed to be absent in ill or debilitated animals (Greenwood, 1986). It should, therefore, be sensitive to any adverse drug effects. The behaviour commences by the animal fully extending from the shell

vertically. The head then orients to the horizontal surface upon which it has been placed. It then makes an initial contact with this surface using its foot. The wave pattern of foot traction is then used to pull the snail over, and this stops when the snail is completely righted.

#### DESIGN

Snails that were inside their shells after injection were placed in a waxed laboratory dissecting dish with the opening of the shell facing up. The time taken for each snail to right itself was recorded using a stop watch. This was taken to be from the emergence of the snails head until the snail was righted and fully extended.

APPLICATION OF THE ABOVE TESTS TO SCREEN PROTEIN SYNTHESIS  
INHIBITORY DRUGS FOR BEHAVIOURAL SIDE-EFFECTS

EXPERIMENT 1

OPEN FIELD INVESTIGATIONS OF DEBILITATING EFFECTS OF  
ANISOMYCIN, ACTINOMYCIN D, PUROMYCIN, AND CYCLOHEXIMIDE.

Two open field investigations were conducted on 3 doses of each drug at 2 volumes of injection. The doses employed were 0.00025mg/ml, 0.025mg/ml, and 2.5mg/ml. The initial study investigated their effects 10 minutes after injection, and the second study screened for effects 12 hrs post injection. The 2 volumes of drug injected were 0.1ml and 0.3 ml. Three hundred snails were employed. Ten snails were used in each condition in each experiment. Equivalent saline conditions were also used to control for post injection trauma. The experiments were conducted between 2400 and 1430 hrs on the mornings of January 28, 29, and 30, 1985, and February 1 and 2, 1986 Temperature in the laboratory varied between 17 C and 22 C.

RESULTS FOR 0.1 ml INJECTIONS

A 3-way ANOVA, with between subject factors of dose, drug, and retest, revealed significant effects of dose ( $F(.2/270) = 41.374, p < .001$ ), drug ( $F(4/270) = 48.425, p < .001$ ), and retest ( $F(1/270) = 57.430, p < .001$ ), with significant interactions of Dose x Drug ( $F(8/270) = 8.381,$

$p < .001$ ), Dose x Test ( $F(2/270) = 6.850$ ,  $p < .001$ ) and Drug x Test ( $F(4/270) = 19.314$ ,  $p < .001$ ). The results for all doses in this and subsequent experiments in this chapter are presented graphically.

#### DOSE 1 (0.00025mg/ml)

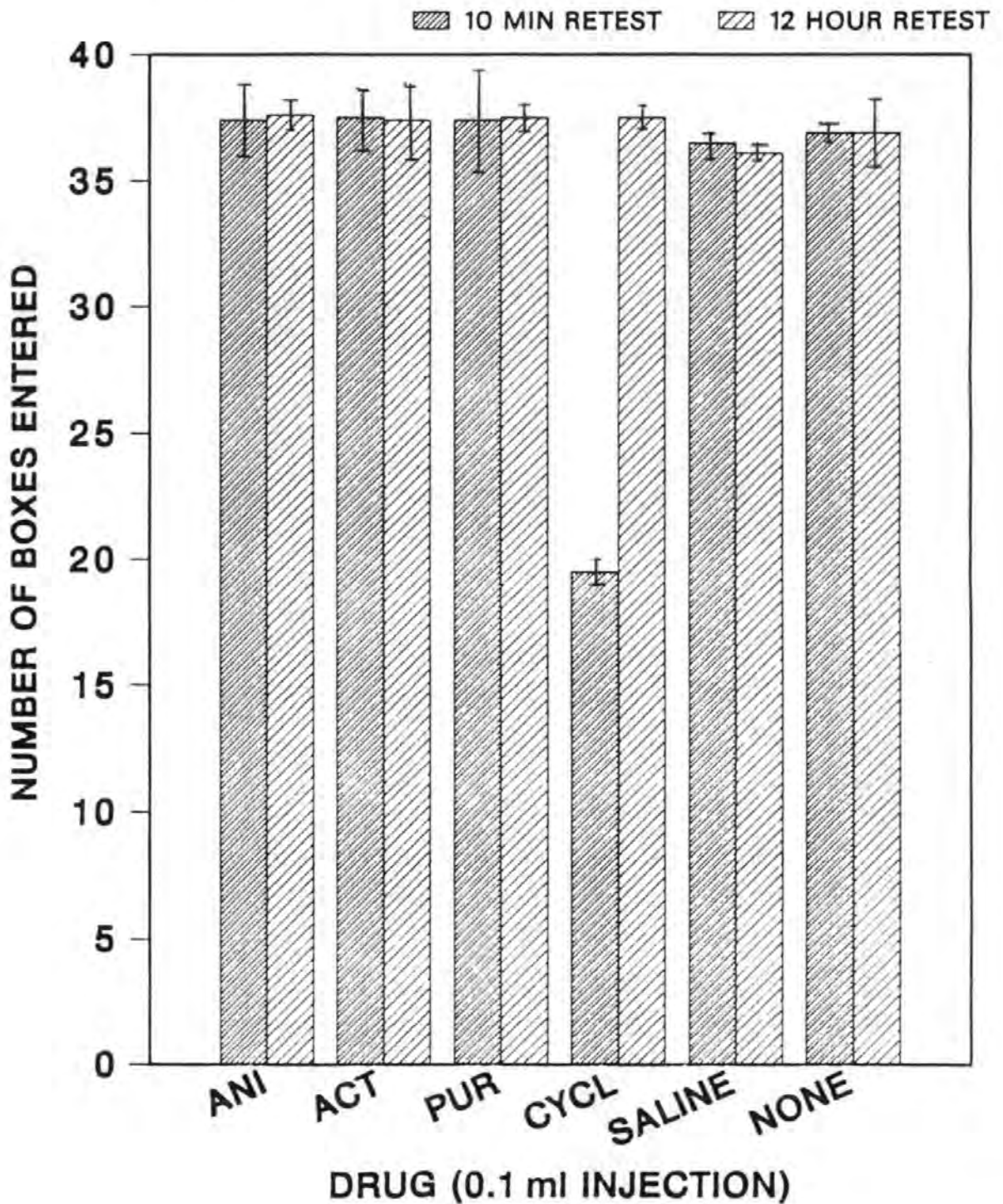
With a 0.1ml injection screened at 10 mins post injection, there appeared to be no deficit in ambulatory behaviour compared to saline with the antibiotics anisomycin, actinomycin D, or puromycin. However, cycloheximide did produce decreased ambulation compared with saline control and no treatment groups. No snails displayed any effect of any of the drugs on levels of ambulation at 12 hrs post injection. The results for Dose 1 are illustrated in Figure 8.1.

#### DOSE 2 (0.025mg/ml)

Again, there was no effect of anisomycin, actinomycin D, or puromycin at either 10 mins or 12 hrs post injection compared to saline controls or no treatment group. Ten minutes post injection there was evidence of decreased ambulation in the cycloheximide condition, but this had recovered in the 12 hr test to control levels. These results are shown in Figure 8.2.

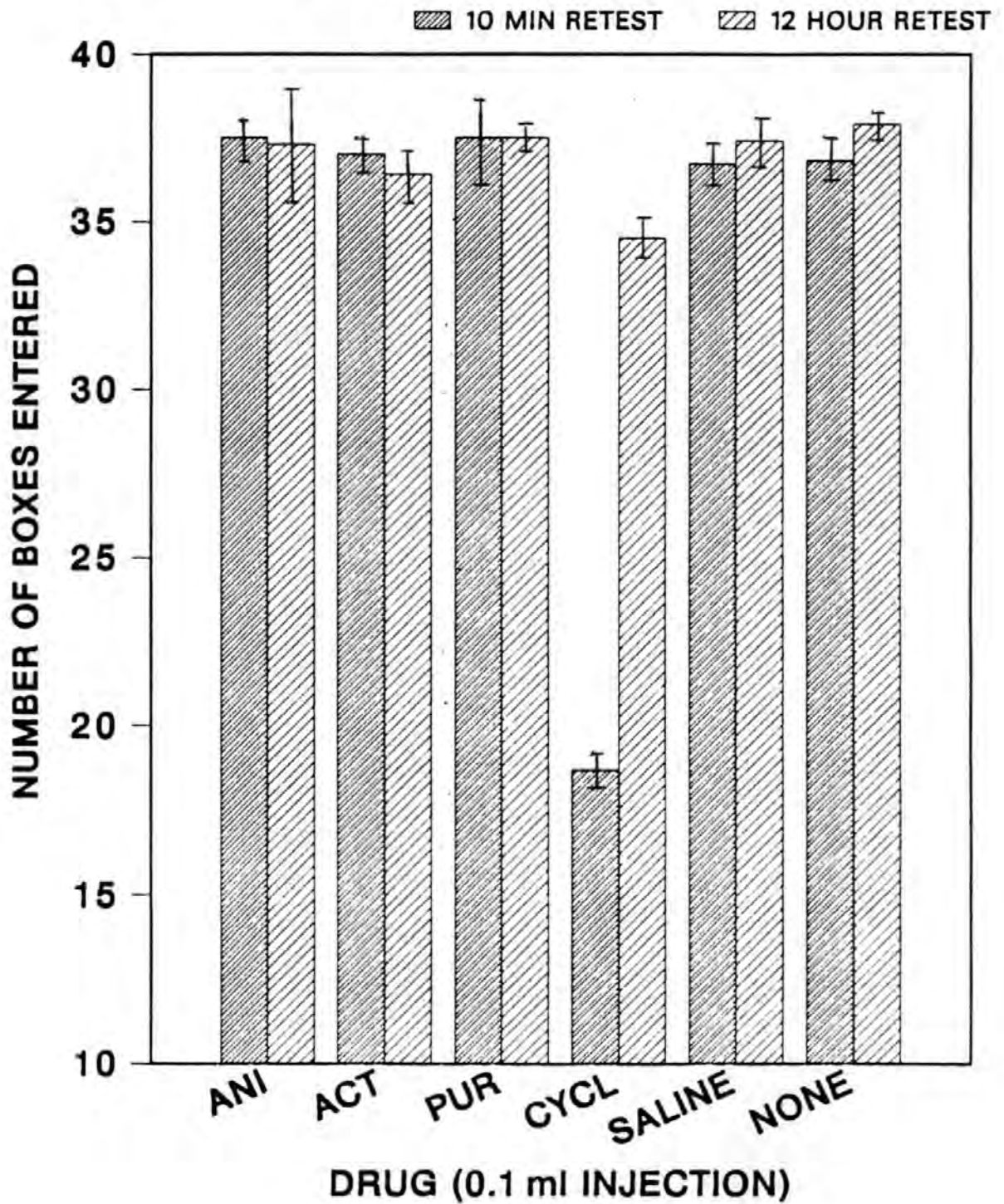
FIGURE 8.1

DRUG EFFECTS IN OPEN FIELD AT DOSE 1, VOL 1  
0.00025 mg/ml



**FIGURE 8.2**

**DRUG EFFECTS IN OPEN FIELD AT DOSE 2, VOL 1**  
0.025 mg/ml



### DOSE 3 (2.5mg/ml)

While anisomycin appeared to have no effect on this test at this dosage in the 10 mins or 12 hrs test, all other drugs showed a deficit in ambulation in the 10 mins test. In the 12 hrs test, actinomycin D and puromycin had recovered to no treatment and saline control levels, and although cycloheximide showed some recovery, this recovery did not reach the levels shown by the saline controls (see Figure 8.3).

### RESULTS FOR 0.3 ml INJECTIONS

#### DOSE 1

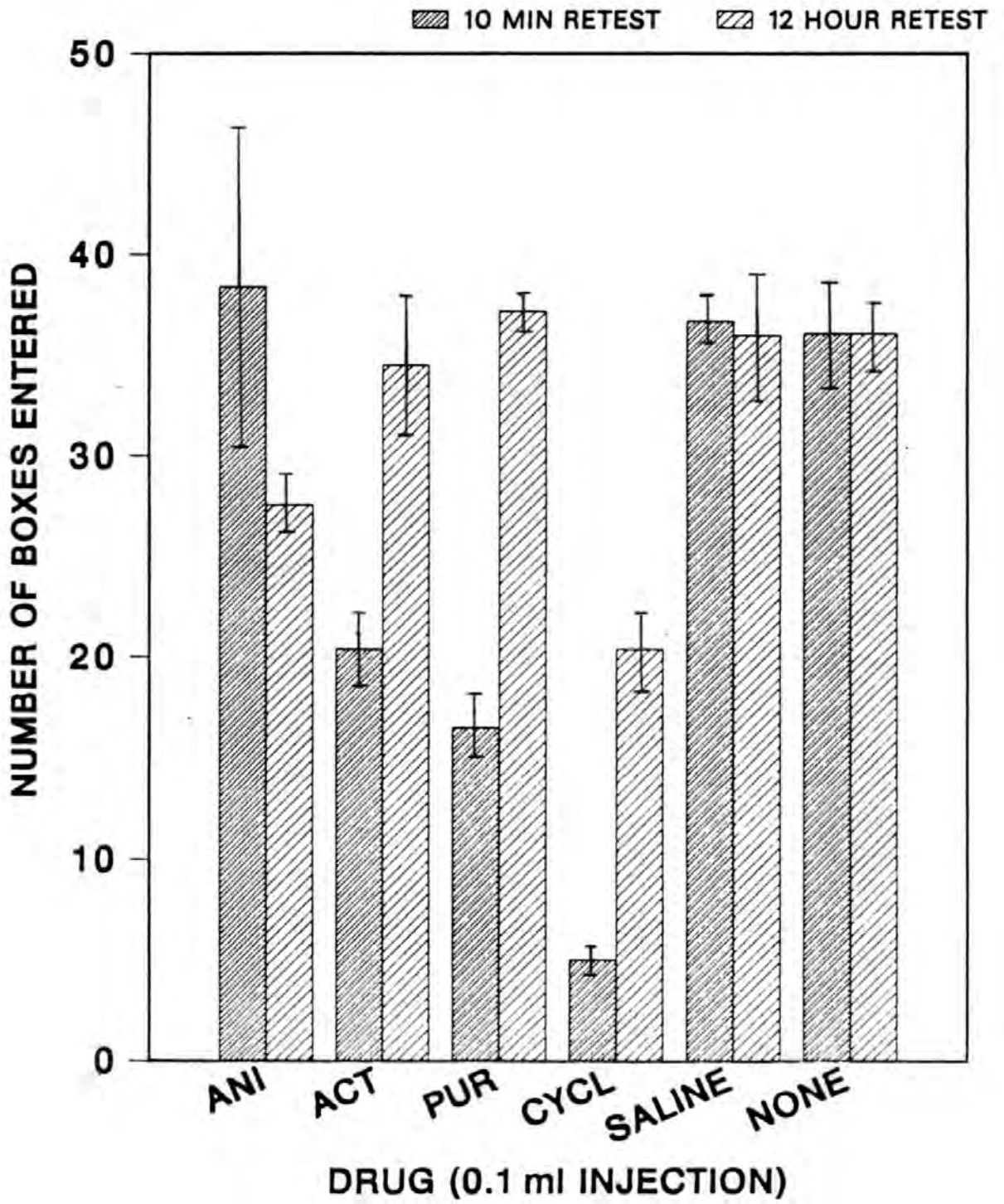
At the 10 mins test, all drugs markedly reduced ambulatory behaviour with cycloheximide reducing movement to virtually zero. It was of note that the levels of the saline controls were also less than no treatment group which suggests that volume of injection itself affects snail behaviour. However, in the 12 hrs test, all conditions had recovered to a level comparable to the no treatment group suggesting that the volume of injection had a debilitating effect on movement initially at this dose, but the snails recovered from this dose by the 12 hrs test. These results are depicted in Figure (8.4).



**FIGURE 8.3**

**DRUG EFFECTS IN OPEN FIELD AT DOSE 3, VOL 1**

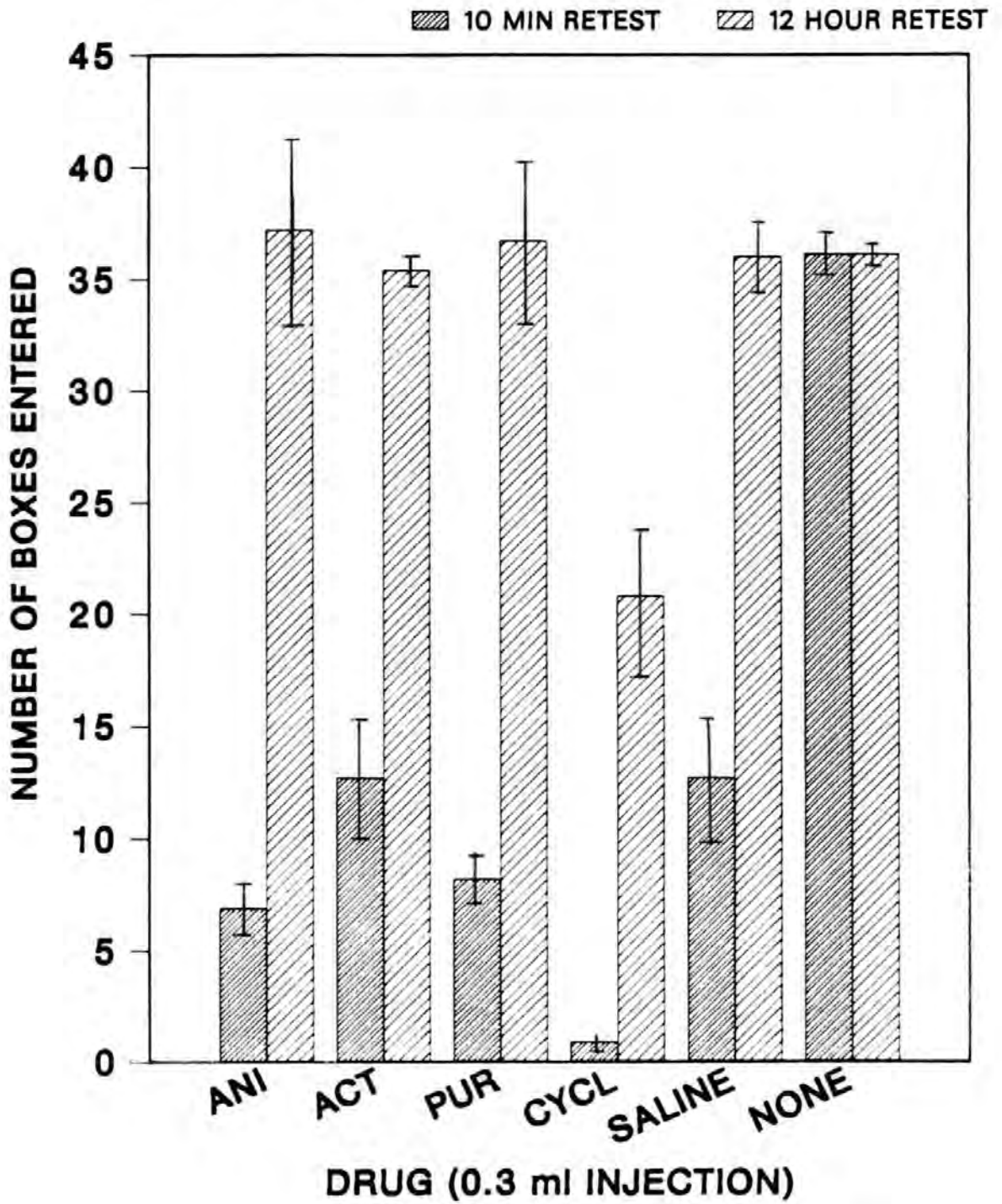
2.5 mg/ml



**FIGURE 8.4**

**DRUG EFFECTS IN OPEN FIELD AT DOSE 1, VOL 2**

0.00025 mg/ml



## DOSE 2

All drugs showed a debilitating effect on ambulation in the 10 minutes post injection test. The saline condition also showed a debilitation compared to the no treatment group. In the 12 hrs test, the anisomycin and saline groups showed a level of ambulation that was similar to the no treatment group. The levels of movement in the other drug conditions also showed some increase in ambulation, but the levels were still lower than no treatment controls (see Figure 8.5).

## DOSE 3

Compared with the no treatment condition, the levels of ambulation were negligible in all drug conditions, and there was no movement detectable in the cycloheximide condition at the 10 mins post injection test. Again, the saline group also showed a decrement in ambulation compared to no treatment controls. In the 12 hrs test, there was no recovery of ambulation levels in any of the drug groups, and the cycloheximide group were all dead (see Figure 8.6).

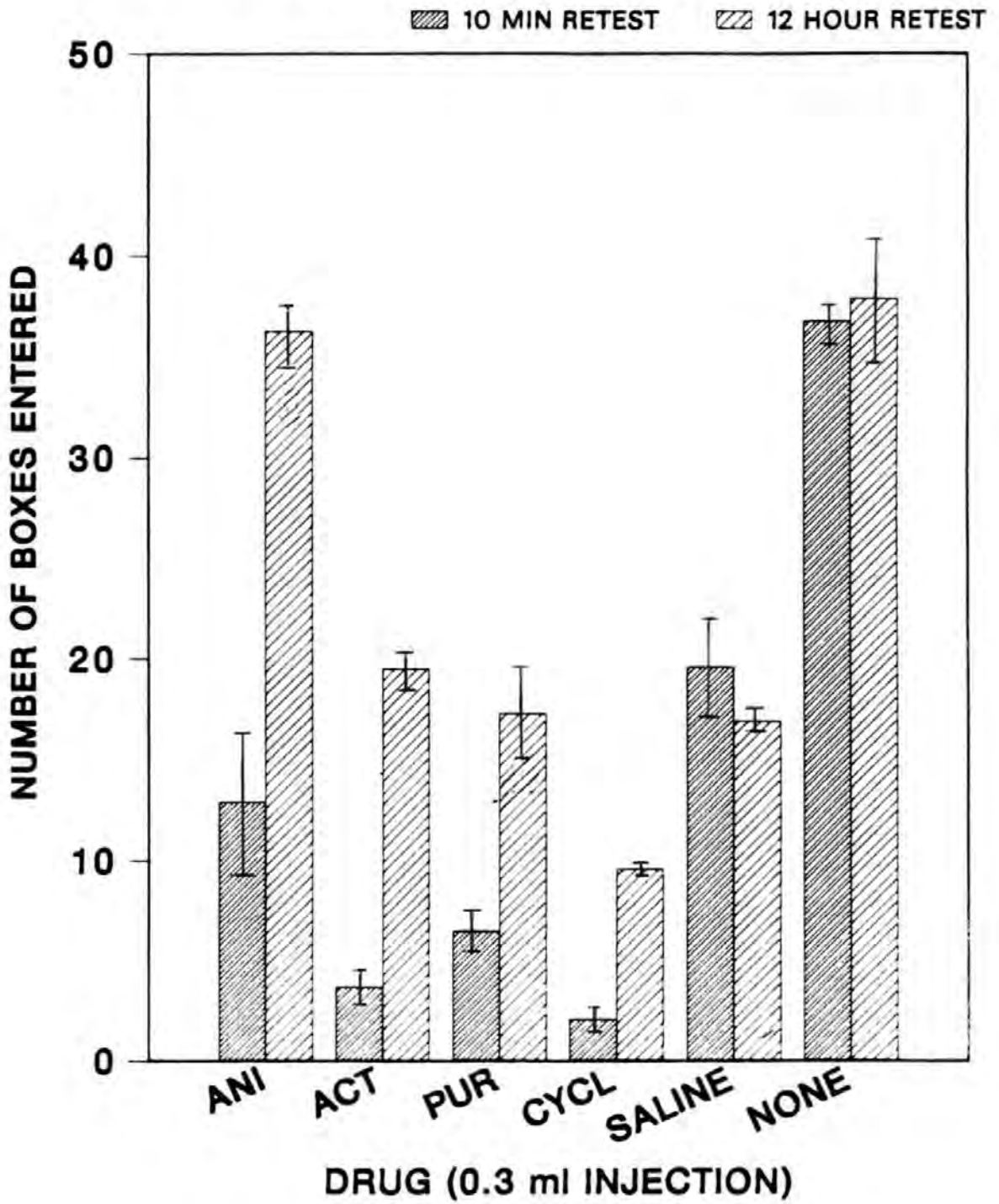
## CONCLUSIONS

From the above two studies utilising the open field paradigm, it was concluded that Cycloheximide had such a debilitating effect on snail movement that it was not suitable for inclusion in the learning studies. Also, the

**FIGURE 8.5**

**DRUG EFFECTS IN OPEN FIELD AT DOSE 2, VOL 2**

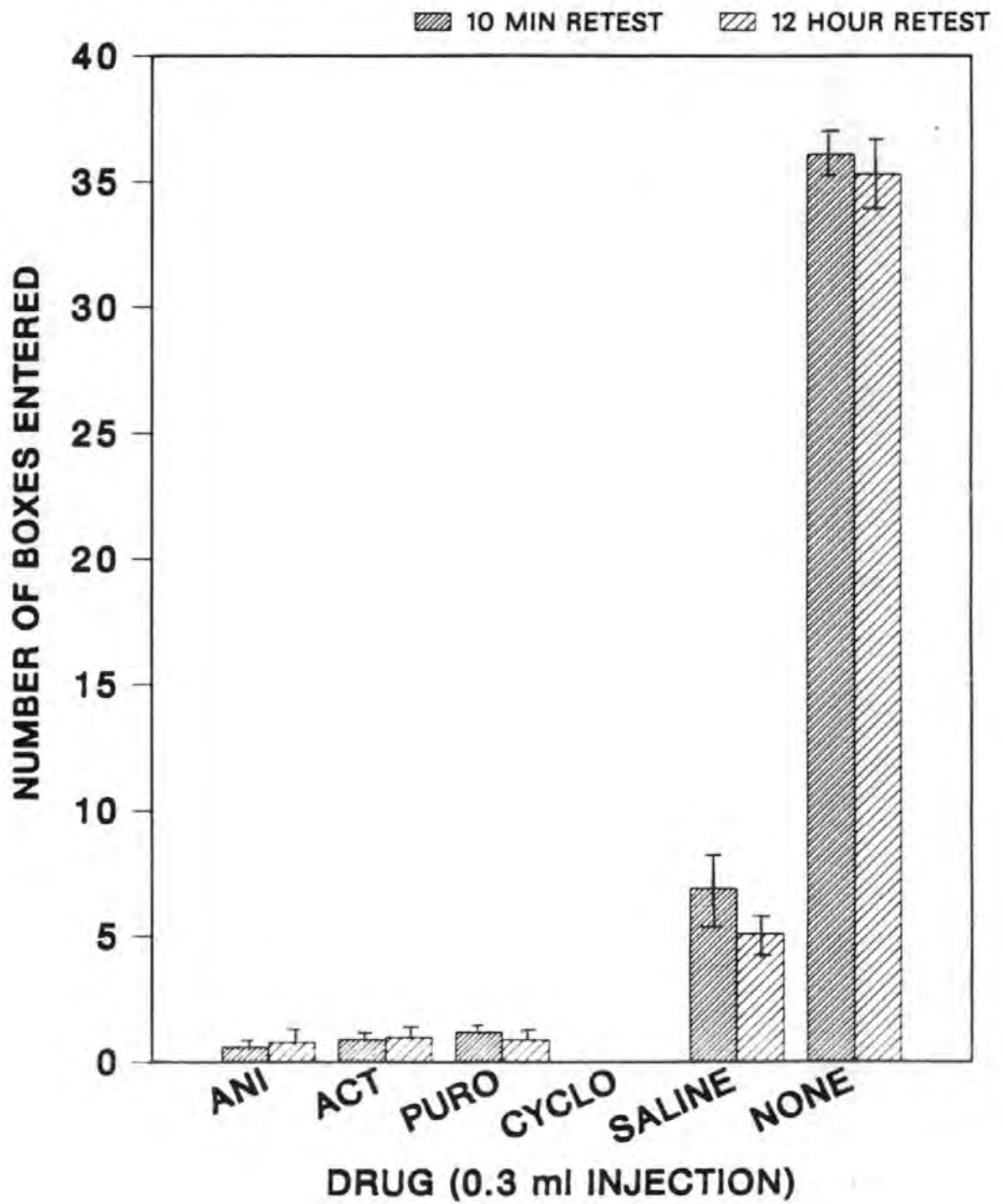
0.025 mg/ml



**FIGURE 8.6**

**DRUG EFFECTS IN OPEN FIELD AT DOSE 3, VOL 2**

2.5 mg/ml



results suggest that the volume of drug to be administered should be 0.1 ml rather than 0.3 ml, as the latter volume appeared to have a debilitating effect even with injections of saline. All the remaining antibiotics appeared suitable from these tests to continue on the test battery analysis for side effects at the dosage levels of 1 and 2. Dose 3 was also further investigated in light of the fact that its debilitation dissipated with time.

## EXPERIMENT 2

### AN INVESTIGATION OF THE EFFECTS OF ANISOMYCIN, ACTINOMYCIN D AND PUROMYCIN ON THE WITHDRAWAL REFLEX

The effects of these antibiotics are reported for a 0.1 ml injection of the same three doses, screening for effects at 10 mins and 5 hrs post training. Two tests are included for this behaviour, 1) response to touch, and 2) the placement test (see tests section). One hundred and fifty snails were tested at each post test interval (10 mins and 5 hrs post-injection) with 10 snails in each group, 5 groups per dose, one group per drug (actinomycin D, anisomycin, and puromycin), a saline control, and a no injection group. These experiments were conducted between 2430 and 01030 Hrs on the mornings of February 3, 4, 5, 6, 7, and 8, 1986. Temperature in the laboratory varied from 15 to 19 C.

## RESULTS TOUCH TEST

A 3 way ANOVA on response duration data with three between subject factors revealed a significant effect of dose ( $F(2/270) = 13.684, p < .001$ ), drug ( $F(4/270) = 6.465, p < .001$ ), and test ( $F(1/270) = 16.557, p < .001$ ) with a significant interaction of dose x test ( $F(2/270) = 11.966, p < .001$ ). All other interactions failed to reach significance ( $p > .5$ ). Further analysis of each dose employed revealed the following effects of the drugs:

### DOSE 1

In the 10 mins post training test, none of the antibiotics interfered with the withdrawal response in terms of duration of response or category of response elicited. There was also no difference on these measures between saline and no injection groups. Similarly, there was no difference between the performance of snails in any of the drug conditions compared to saline or no injection groups in the 5 hrs test. The results in terms of response durations for drugs at Dose 1 are depicted in Figure 8.7.

### DOSE 2

In both the 10 mins and the 5 hrs tests, there was no evidence of any of the three antibiotics drugs debilitating or disrupting withdrawal behaviour. These effects are shown in Figure 8.8.

FIGURE 8.7

DRUG EFFECTS IN WITHDRAWAL TO TOUCH TEST FOR DOSE 1

0.00025 mg/ml

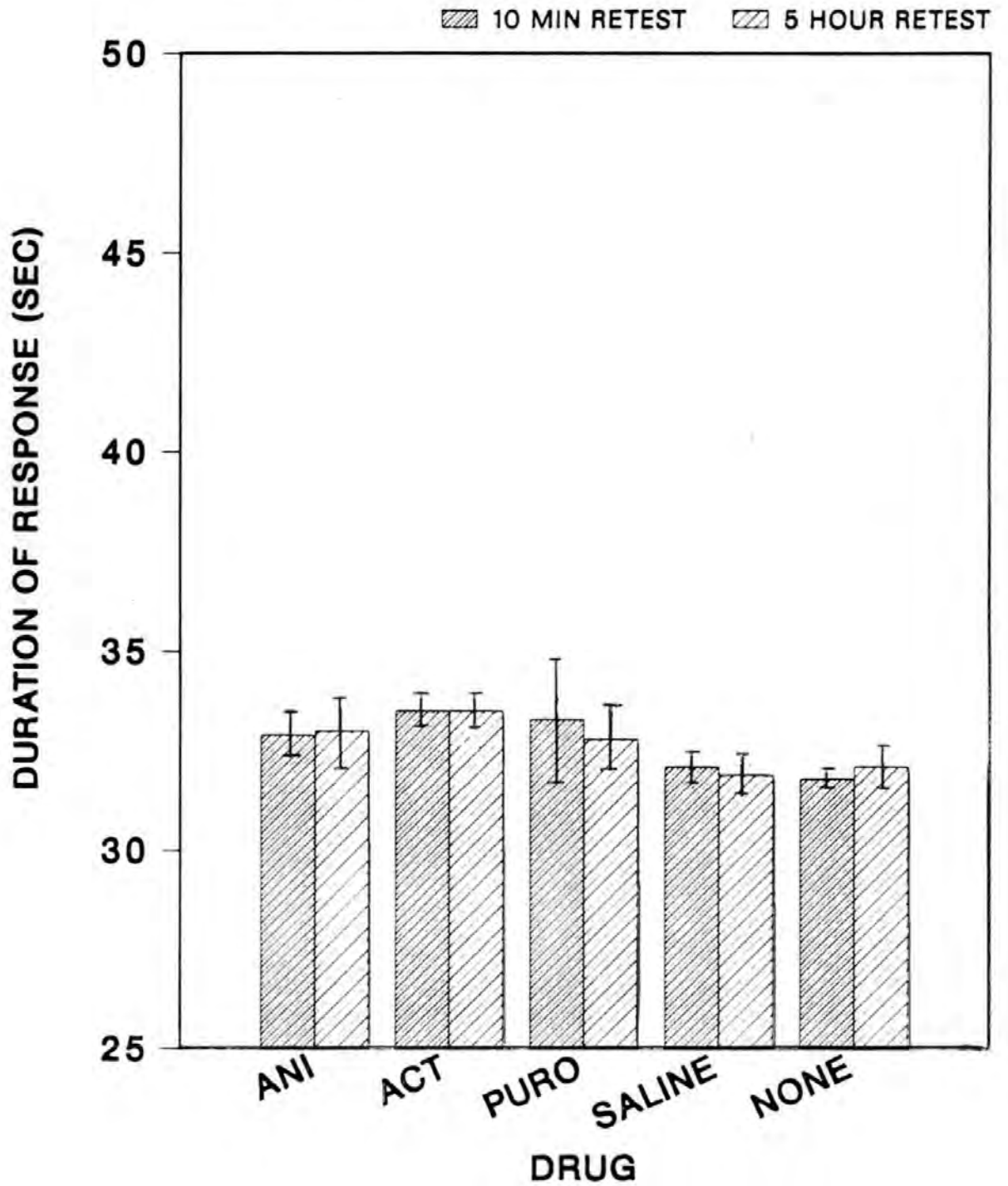
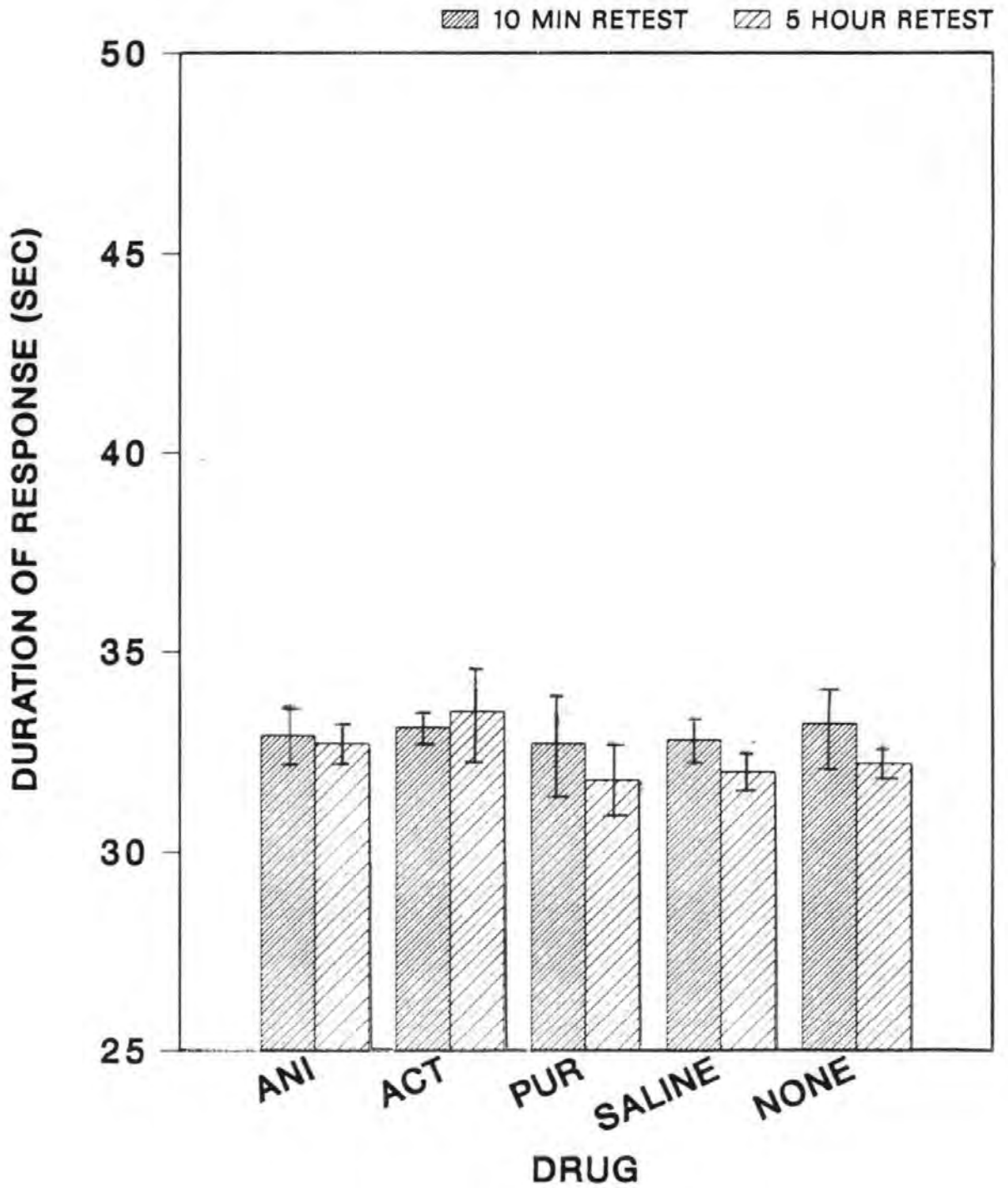




FIGURE 8.8

DRUG EFFECTS IN WITHDRAWAL TO TOUCH TEST FOR DOSE 2  
0.025 mg/ml



### DOSE 3

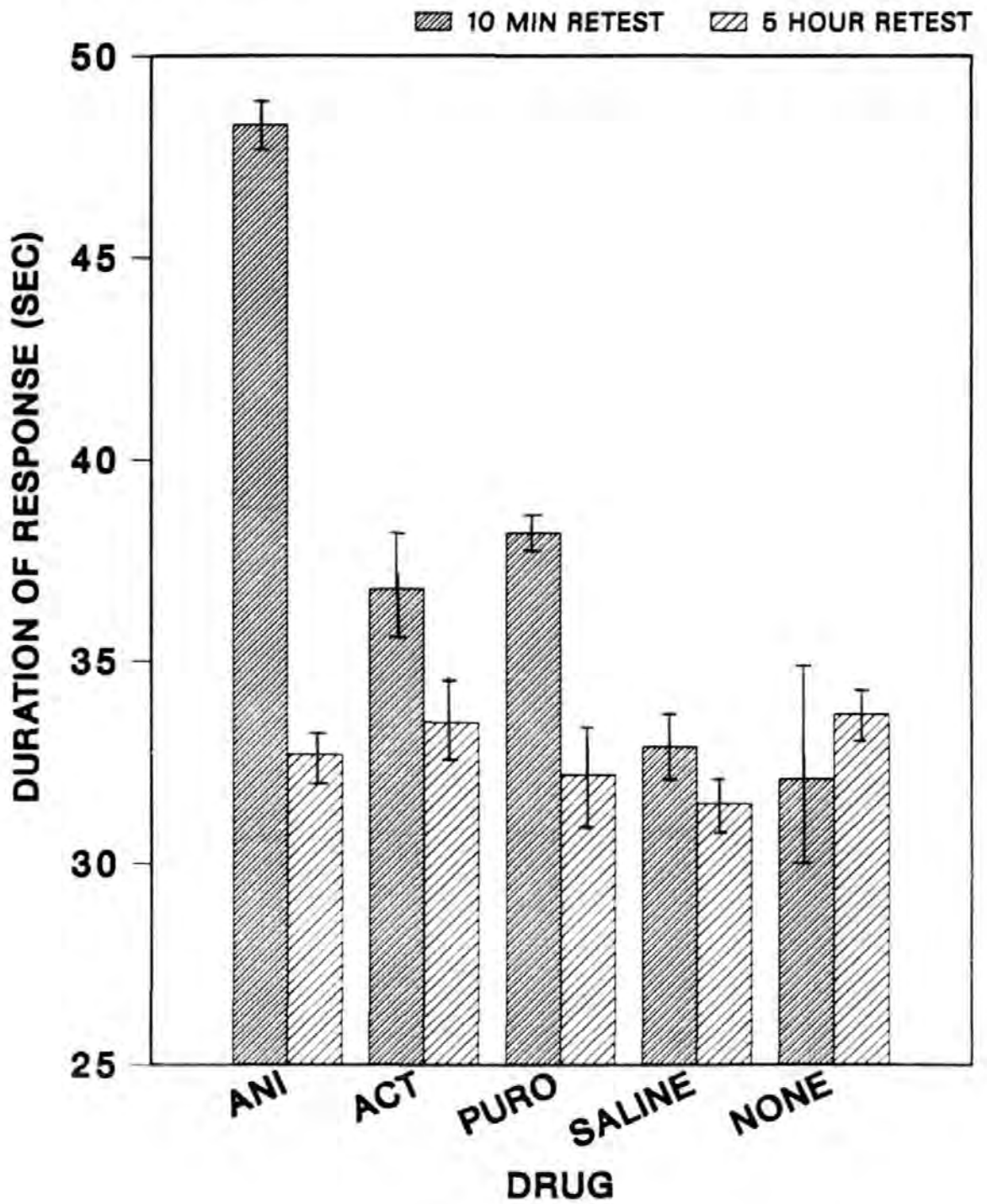
At 10 mins post injection, it was evident that Anisomycin injected snails responded with much larger response durations, with 2 snails failing to emerge from their shells within the allotted time span. Actinomycin D and Puromycin also produced a heightened level of withdrawal response, but snails in these groups showed shorter response durations than the Anisomycin group. Tests conducted 5 hrs post injection showed that the level of responses in all three drug groups did not vary from the saline or no injection condition. The results for this dose are shown in Figure 8.9.

### RESULTS PLACEMENT TEST

Results for snails on this version of the withdrawal reflex test were analysed in a 3-way ANOVA. This showed the same effects as were observed in the above test with a significant effect ( $p < .001$ ) of Dose ( $F(2/270) = 51.407$ ,  $p < .001$ ), Drug ( $F(4/270) = 10.398$ ,  $p < .001$ ), and Test ( $F(1/270) = 43.741$ ). The following interactions were also significant, Dose x Drug ( $F(8/270) = 9.347$ ,  $p < .001$ ), Dose x Test ( $F(2/270) = 42.872$ ,  $p < .001$ ), Drug x Test ( $F(4/270) = 8.244$ ,  $p < .001$ ) and Dose x Drug X Test ( $F(8/270) = 10.663$ ,  $p < .001$ ).

**FIGURE 8.9**

**DRUG EFFECTS IN WITHDRAWAL TO TOUCH TEST FOR DOSE 3**  
**2.5 mg/ml**



#### DOSE 1

There was no effect of either actinomycin D, anisomycin or puromycin at this dose compared to saline or no injection control groups. This was the case at both the 10 mins and the 5 hrs tests. These results concur with those of the withdrawal to touch test above. The results for the five groups at this dose are depicted in Figure 8.10.

#### DOSE 2

Again, there was no difference in either test between antibiotic treated snails and those in the saline or no injection condition (see Figure 8.11).

#### DOSE 3

At this dose, anisomycin treated snails showed greater response durations than saline and no injection treated snails at the 10 mins retest. However, this effect was no longer in evidence at the 5 hrs test. Similarly, in the earlier test, actinomycin D and puromycin caused greater response durations than those exhibited by saline and no treatment conditions and this difference was no longer evidenced 5 hrs post injection. These results are shown in Figure 8.12.

FIGURE 8.10

DRUG EFFECTS IN WITHDRAWAL TO PLACEMENT TEST FOR DOSE 1  
0.00025 mg/ml

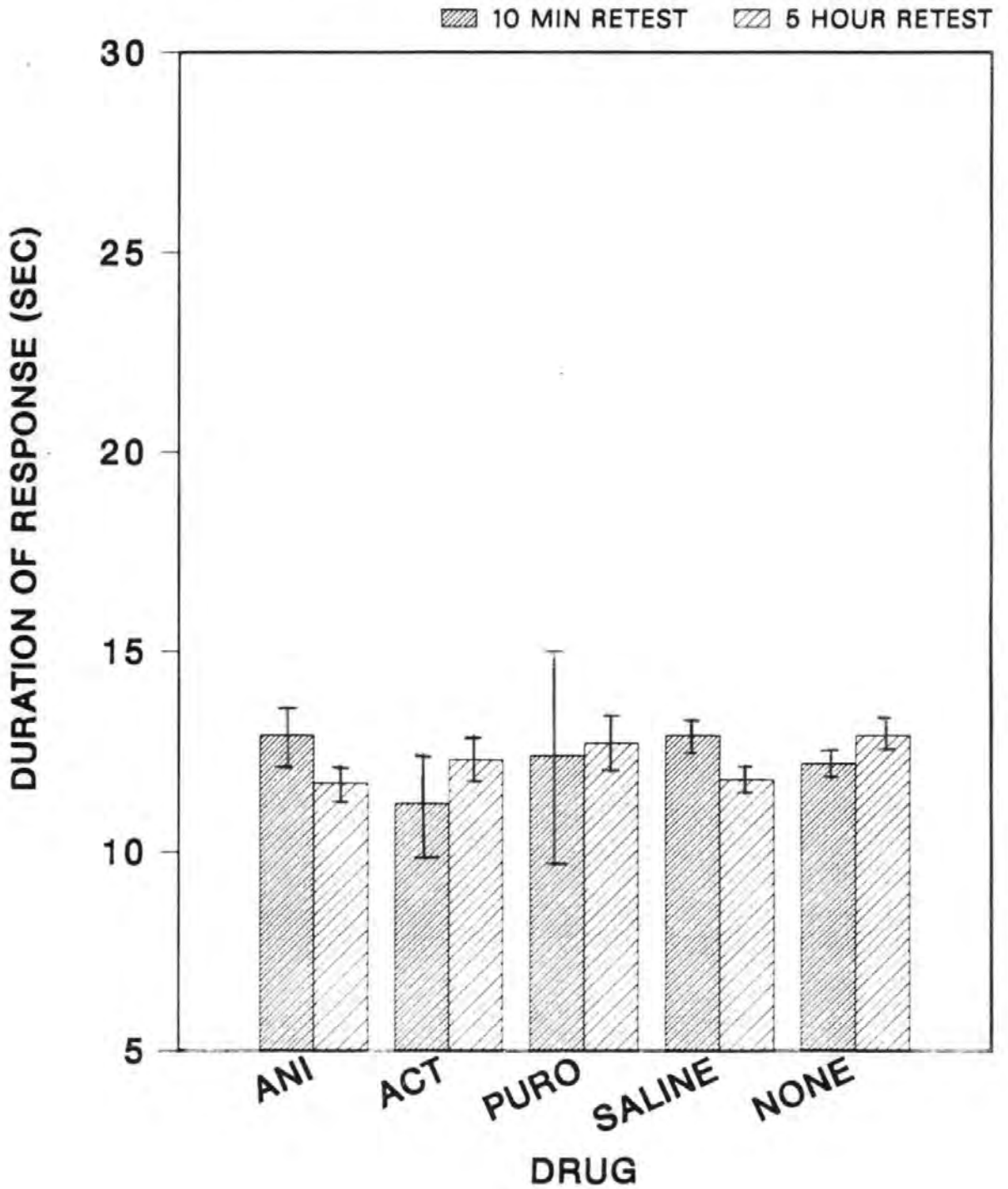
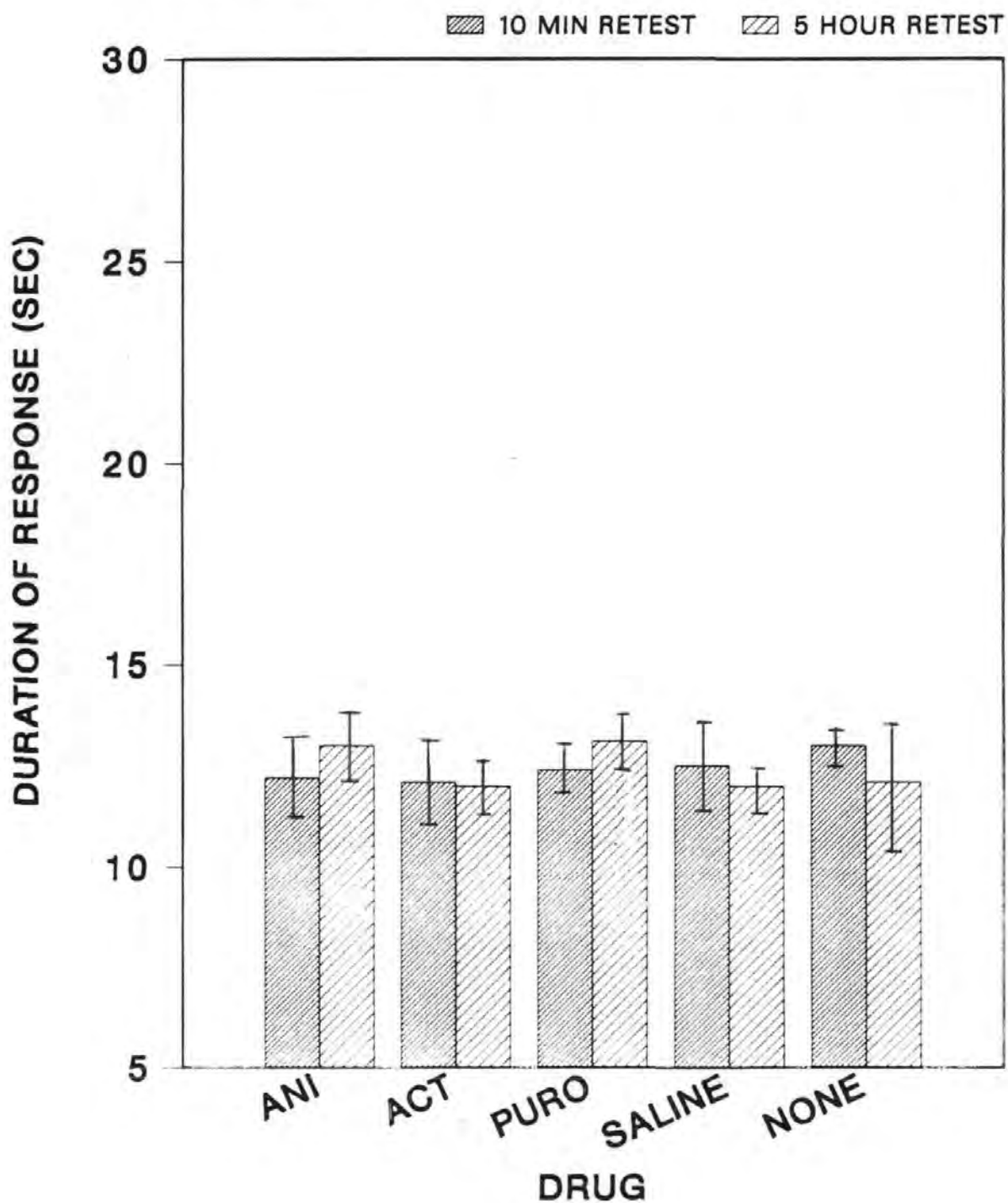


FIGURE 8.11

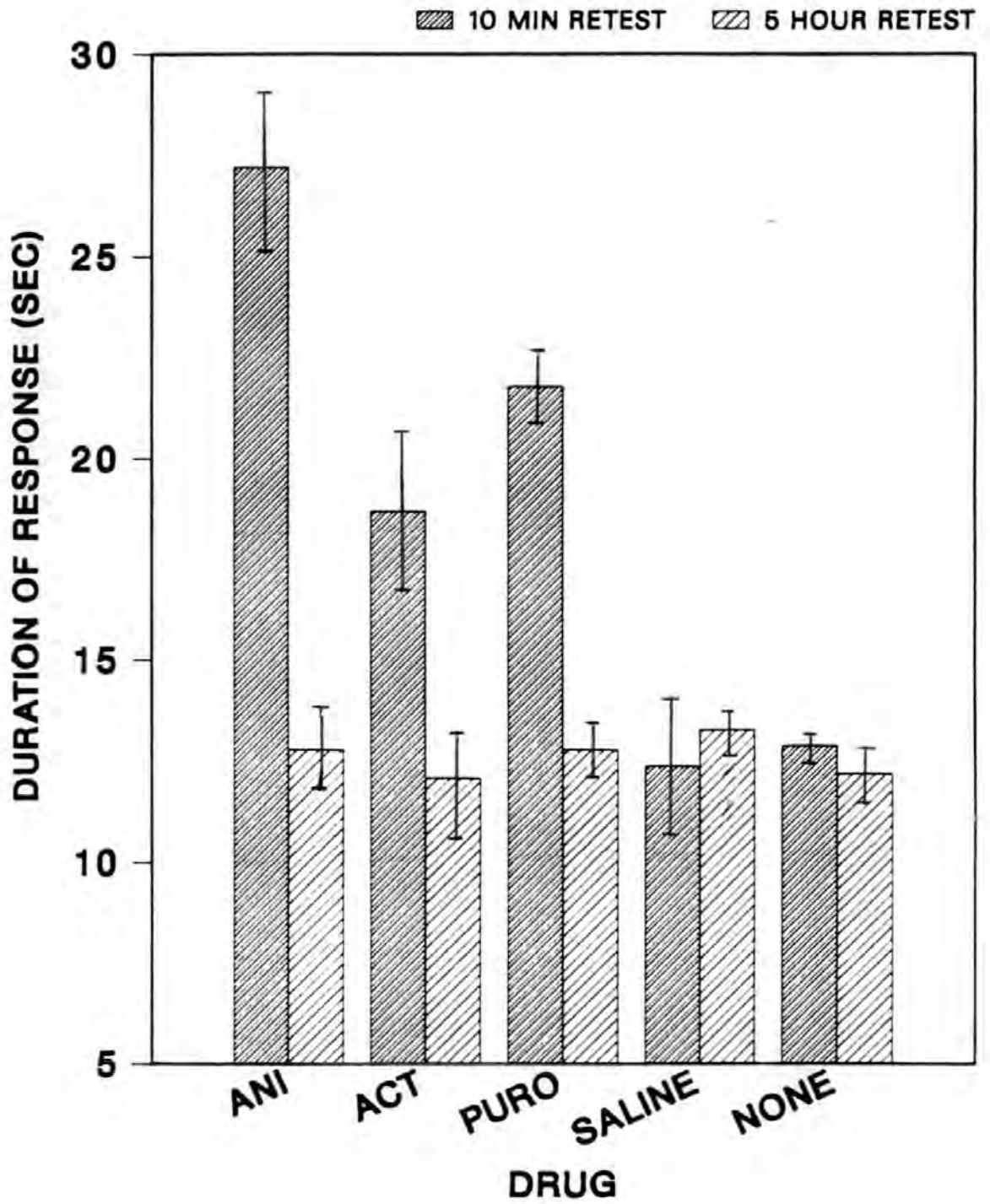
DRUG EFFECTS IN WITHDRAWAL TO PLACEMENT TEST FOR DOSE 2

0.025 mg/ml



**FIGURE 8.12**

**DRUG EFFECTS IN WITHDRAWAL TO PLACEMENT TEST FOR DOSE 3  
2.5 mg/ml**



## CONCLUSIONS FOR TOUCH AND PLACEMENT TESTS

The three antibiotics anisomycin, actinomycin D, and puromycin had no effect on either test at Dose 1 and 2 in both the placement and touch tests. At Dose 3, however, all drugs produced abnormal responses compared to saline and no injection control groups in the 10 mins post injection tests of both touch and placement induced antennae withdrawal. Interestingly, this abnormal responding had recovered by the 5 hrs tests. Consequently, anisomycin, actinomycin D and puromycin proceeded to be tested in Experiment 3 at the above doses. It was concluded, from Experiment 2, that the three antibiotics had no effect on withdrawal or propensity to withdraw at Dose 1 and 2.

## EXPERIMENT 3

### THE EFFECTS OF 3 DOSES OF ANISOMYCIN, ACTINOMYCIN D AND PUROMYCIN ON LEVELS OF GENERAL ACTIVITY

Fifty snails in each of three drug conditions, saline, and no injection control groups were screened for each dose at hourly intervals for 12 hrs. The number active from each group was recorded for each observation. Snails were classified as active if they were extended from shell and displayed locomotion. All snails were housed together in test apparatus (see Tests section). The experiment was conducted on February 10, 1986. Temperature in the laboratory varied between 16 and 20 C.



## RESULTS

The results of this experiment are shown in Figures 8.13 to 8.15. It was evident that, in all 12 observations, there was no effect of any of the 3 antibiotics at either dose 1 or dose 2 in comparison to either saline or no injection groups. Dose 3 of Anisomycin also showed no difference in all observations of activity levels. However, at this dose, the levels of observed activity was radically decreased in both Actinomycin D and Puromycin groups for the first 6 hrs post injection. The level of activity in the Puromycin group had returned to normal after 7 hrs. Activity in the Actinomycin D group gradually increased until it reached an equivalent level to the saline and no injection groups after 10 hrs.

## CONCLUSIONS

At Dose 1 and 2, there was no effect of either anisomycin, actinomycin D, or puromycin on activity levels of the snails. Dose 3 was shown to decrease snail activity levels for actinomycin D and puromycin. There was no effect of anisomycin at dose 3.

FIGURE 8.13

DRUG EFFECTS AT DOSE 1 ON NUMBER OF SNAILS ACTIVE FROM A POPULATION OF FIFTY

0.00025 mg/ml    ANISOMYCIN    SALINE  
                         ACTINOMYCIN D    NO INJECTION  
                         PUROMYCIN

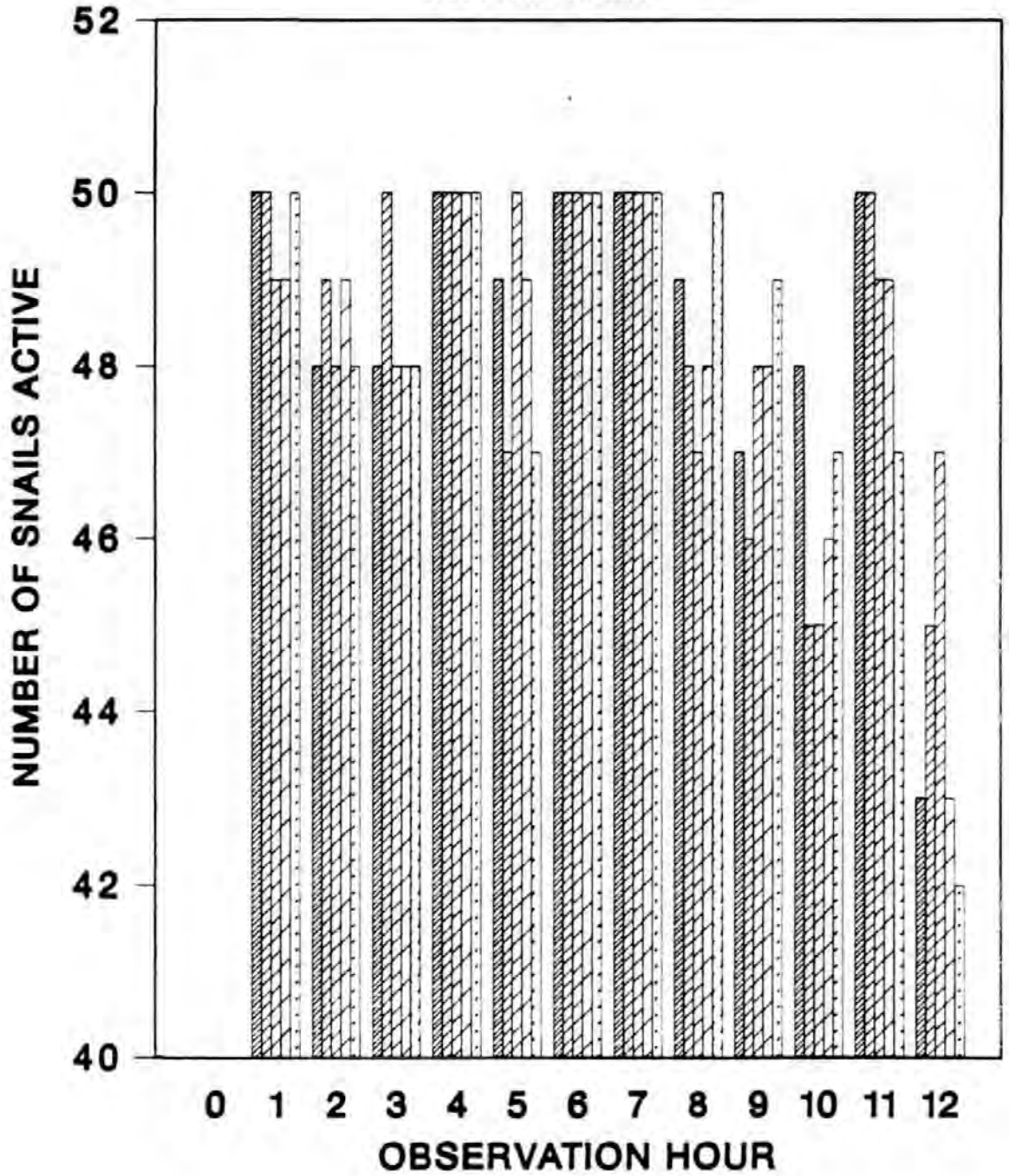


FIGURE 8.14

DRUG EFFECTS AT DOSE 2 ON NUMBER OF SNAILS ACTIVE FROM A POPULATION OF FIFTY

0.025 mg/ml

ANISOMYCIN

SALINE

ACTINOMYCIN D

NO INJECTION

PUROMYCIN

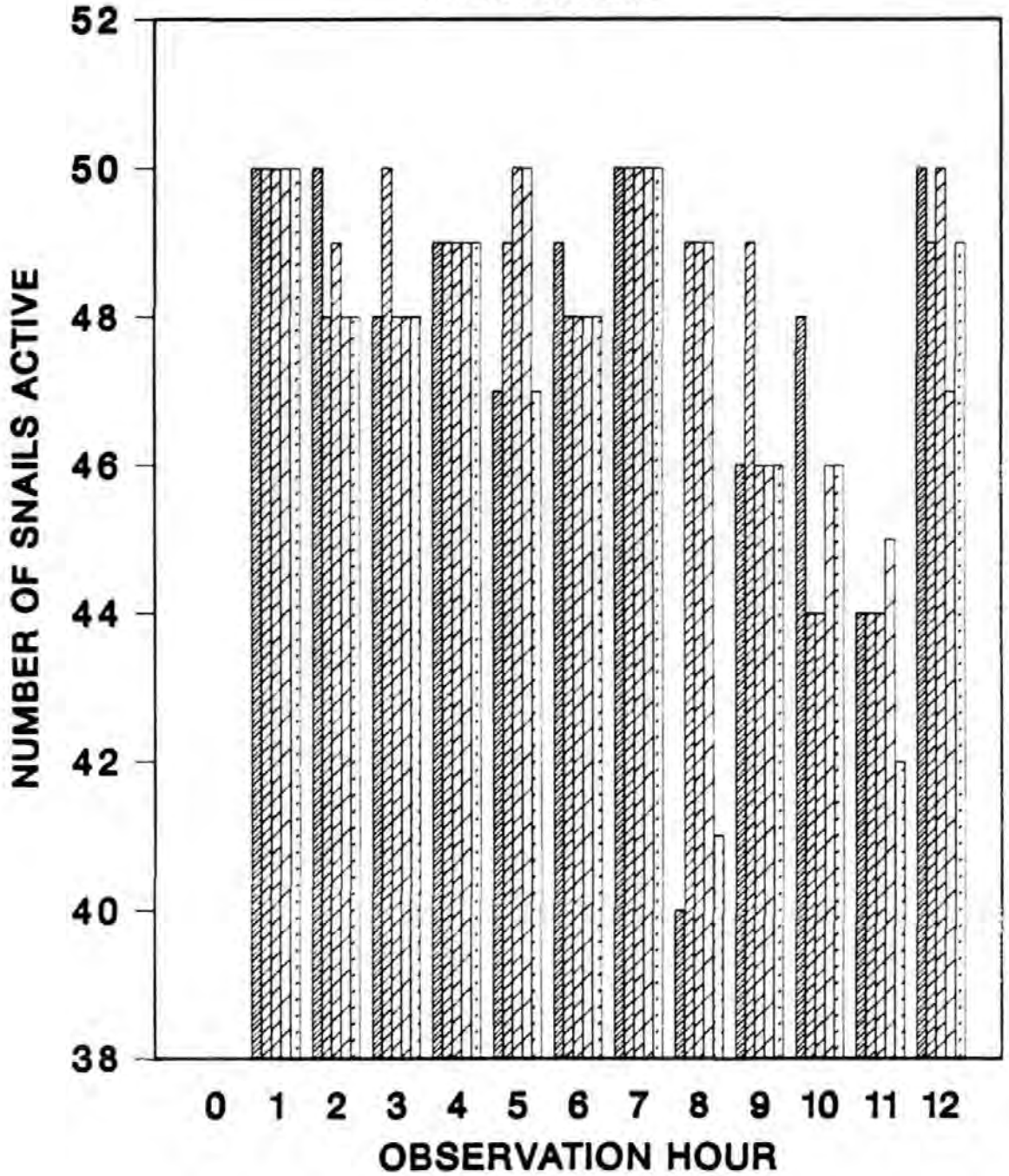





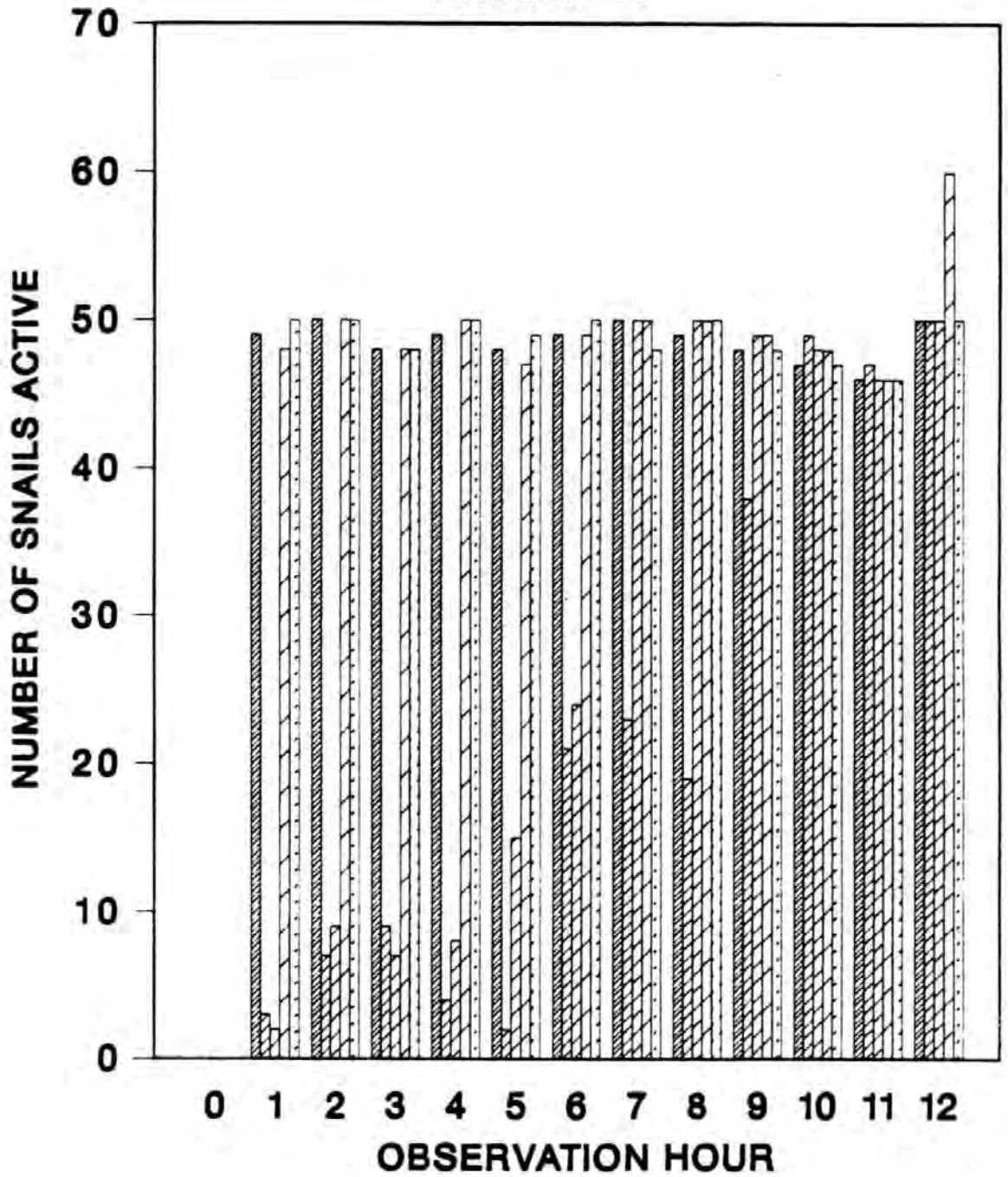


FIGURE 8.15

DRUG EFFECTS AT DOSE 3 ON NUMBER OF SNAILS ACTIVE FROM A POPULATION OF FIFTY

2.5 mg/ml

-  ANISOMYCIN
-  SALINE
-  ACTINOMYCIN D
-  NO INJECTION
-  PUROMYCIN



## EXPERIMENT 4

### THE EFFECTS OF THREE DOSES OF ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN ON THE OLFACTORY CAPABILITIES OF THE SNAIL

Drug effects on the snail's most important sensory modality, olfaction, were screened in the hidden food source test (see Test section). Fifteen groups of 10 snails each were used in a 10 mins test and the same number in a 5 hrs post injection test. They consisted of anisomycin, actinomycin D, puromycin, saline, and no injection groups for each of the 3 dose levels of the antibiotics. Given that these three doses had been tested for locomotor debilitation effects and that none were found, any difference in duration required to locate food source cannot be explained in terms of impaired motor ability and may, therefore, be attributed to abnormal olfactory ability.

#### RESULTS FOR HIDDEN FOOD TEST

Results for this experiment are shown in Figures 8.16 to 8.18 and were analysed in a 3-way ANOVA with between subjects factors of dose, drug, and retest. This revealed a significant effects of Dose ( $F(2/270) = 148.764, p < .001$ ), and Drug ( $F(4/270) = 30.995, p < .001$ ), with a significant Dose x Drug interaction ( $F(8/270) = 25.504, p < .001$ ). There was no significant difference in performance between retests ( $F(1/270) > 1$ ). All other interactions failed to reach significance ( $p < .5$ ).

#### DOSE 1

There was no effect of either anisomycin, actinomycin D, or puromycin in either post injection test on duration taken to locate hidden food compared to saline and no injection control groups (see Figure 8.16).

#### DOSE 2

No evidence of any debilitation of olfactory capabilities were found for any of the drugs at either the 10 minute or the 5 hour tests (see Figure 8.17).

#### DOSE 3

The snails in the three antibiotic conditions all showed longer durations to locate the food source at the 10 minute test compared with both saline and no injection snails. The longest duration was found with puromycin, and shortest in the anisomycin treated snails. It is of note that, with all three antibiotics at this dose, the increased duration to food source did not decrease by the 5 hour test, (see Figure 8.18).

#### CONCLUSIONS

The three antibiotics did not affect the olfactory capabilities of the snails at Dose levels 1 or 2, but Dose 3

FIGURE 8.16

DRUG EFFECTS AT DOSE 1 ON OLFACTION

0.00025 mg/ml

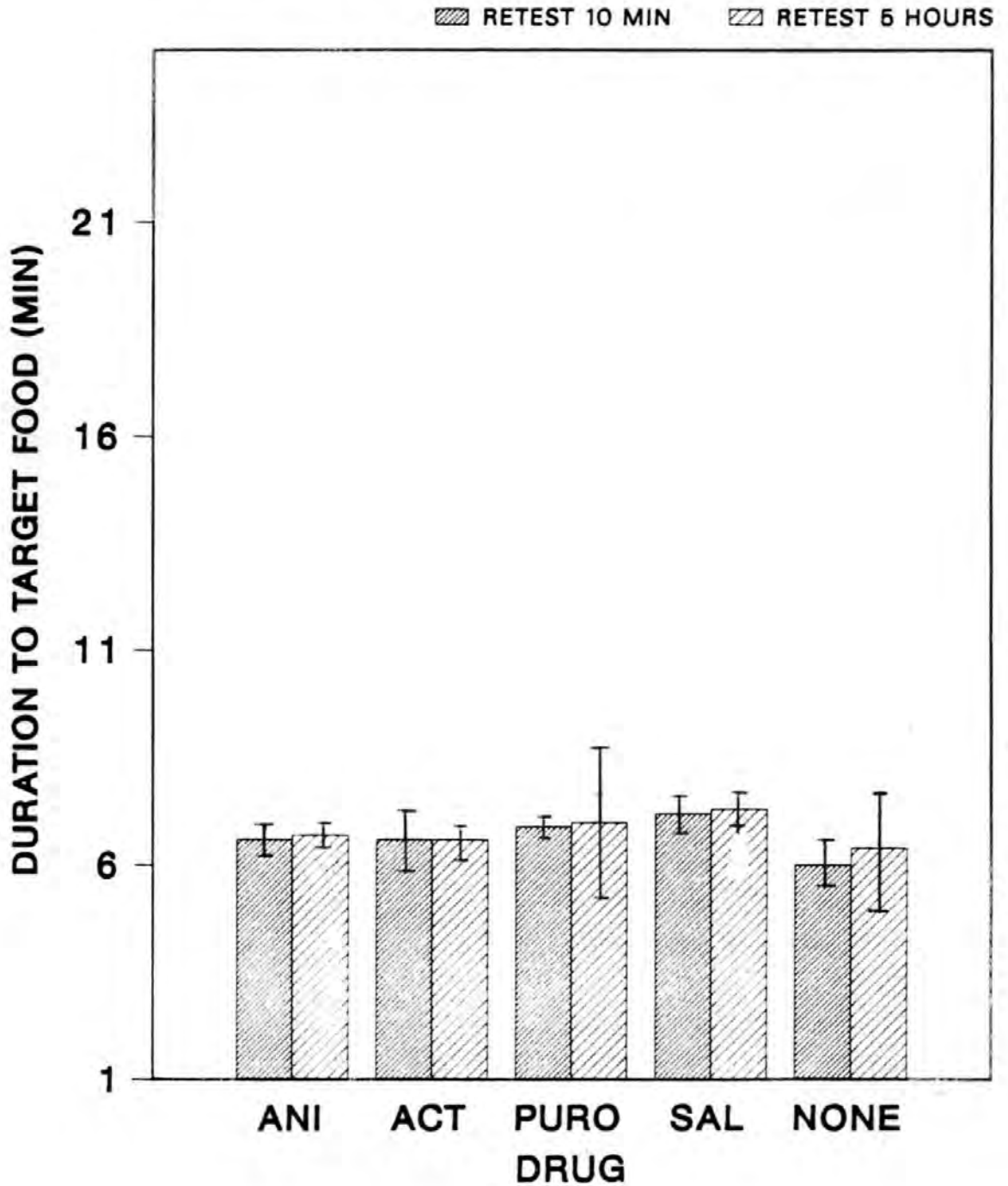


FIGURE 8.17

DRUG EFFECTS AT DOSE 2 ON OLFACTION

0.025 mg/ml

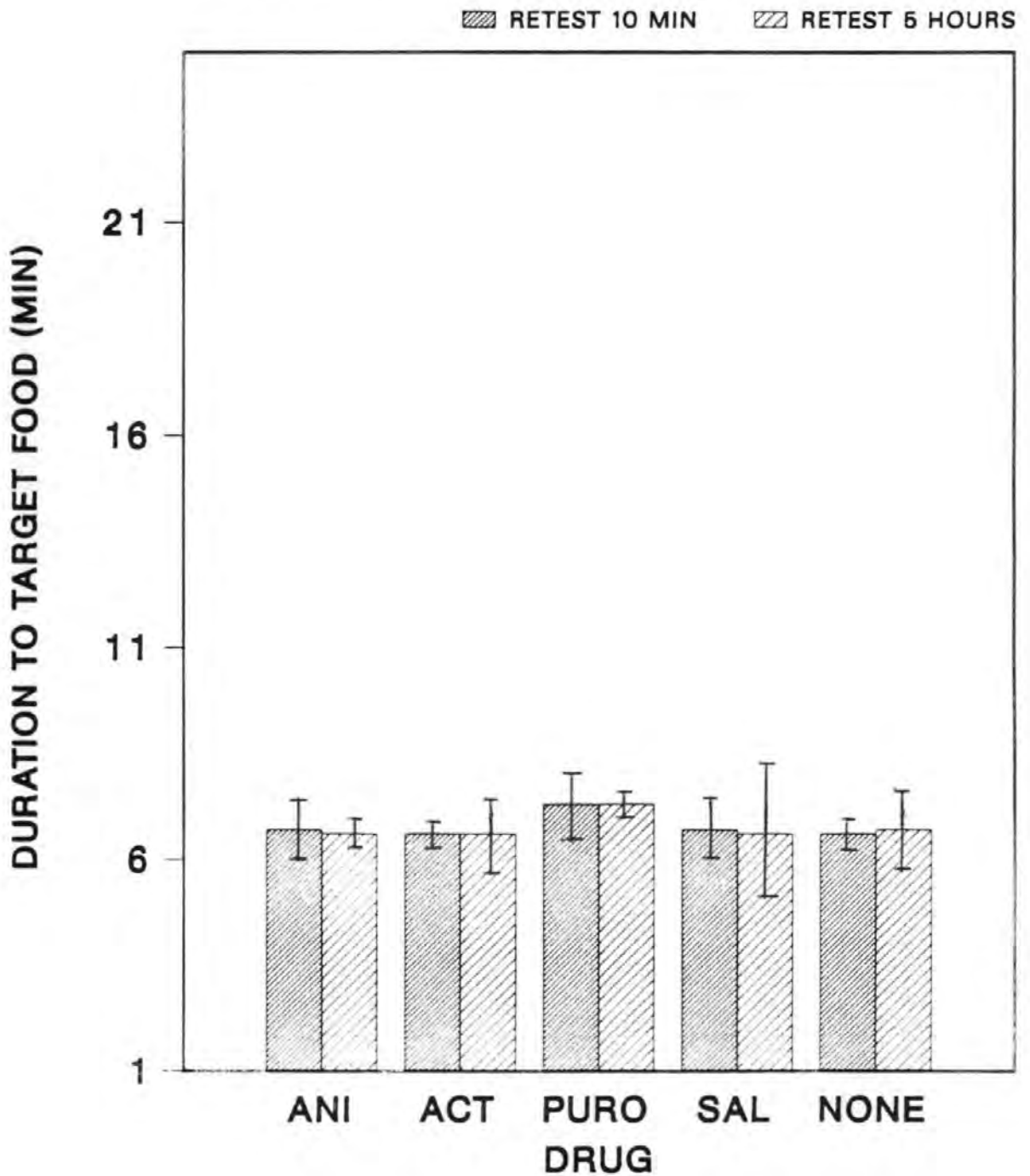
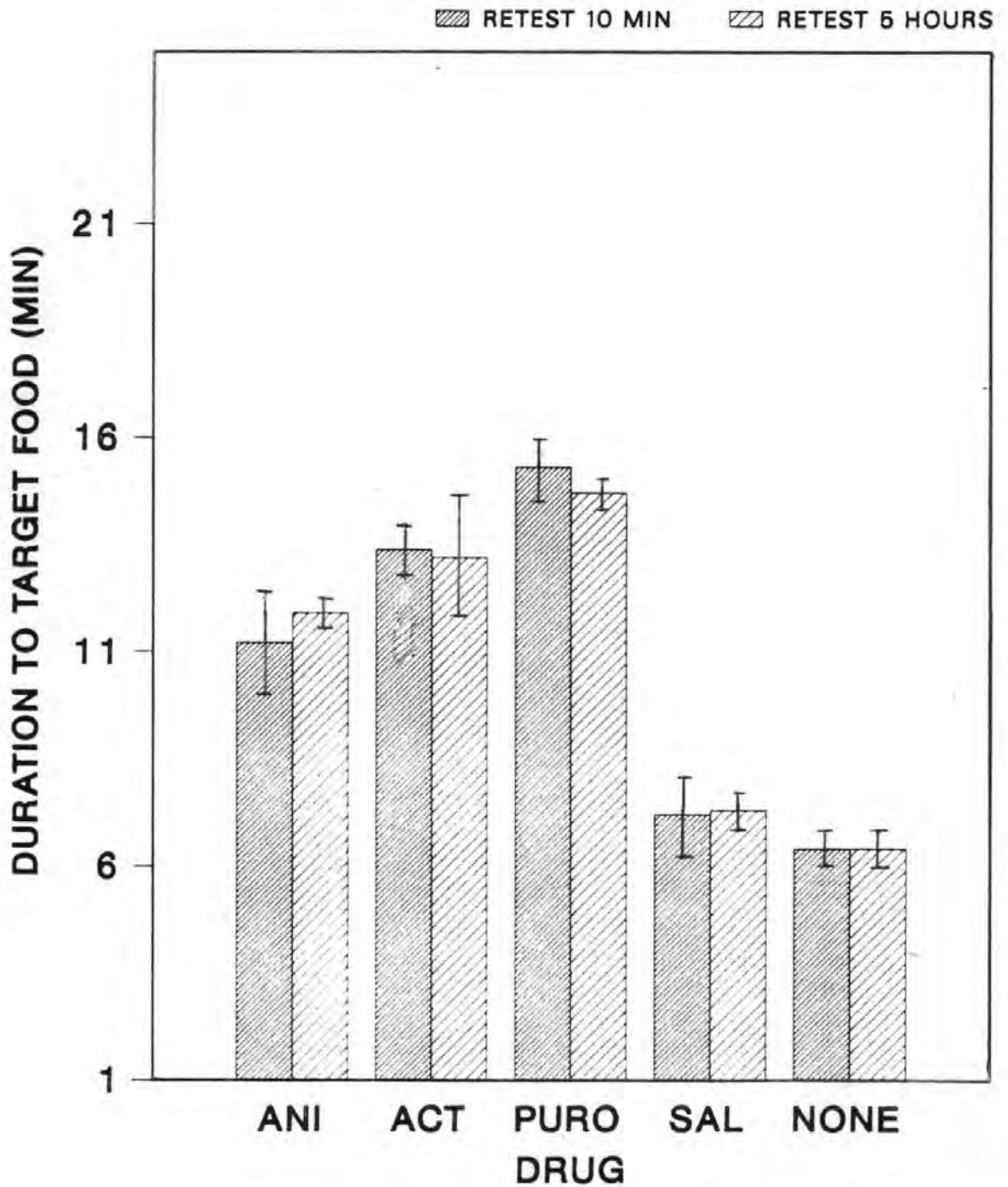




FIGURE 8.18

DRUG EFFECTS AT DOSE 3 ON OLFACTION

2.5 mg/ml



impeded olfaction with all three drug groups and this debilitation was found at 10 mins and 5 hrs post injection. The results with Dose 3 must again question this dose's suitability in learning experiments.

## EXPERIMENT 5

### THE EFFECTS OF THREE DOSES OF ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN ON FEEDING

For each dose there were 20 snails in each of the three drug conditions, the saline, and no injection control groups. Half the animals in each group were tested 10 mins post-injection and half at 5 hrs post-injection. The experiment was conducted between 2400 and 1130 hrs on February 13, 14, 15, and 16, 1986. Temperature in the laboratory varied between 12 and 20 C.

## RESULTS

Analysis in a 3 way ANOVA with between subject factors of drug, dose and retest showed a significant effect of Dose ( $F(2/270) = 87.895, p < .001$ ), Drug ( $F(4/270) = 31.995, p < .001$ ) and no difference between retest ( $F(1/270) < 1$ ). There was a significant dose x drug interaction ( $F(4/270) = 21.211, p < .001$ ). All other interactions failed to reach significance.

#### DOSE 1

There was no effect of any of the three antibiotics on the amount of food consumed compared to the saline or no injection groups at either the 10 mins or 5 hrs test session. There was also no difference between saline and no injection groups. These results are illustrated in Figure 8.19.

#### DOSE 2

Again, there was no difference at either post injection interval between any of the drug groups and the saline or no injection control groups (see Figure 8.20).

#### DOSE 3

In the 10 mins post training test, snails in all three antibiotic conditions showed a decrement in the amount of food consumed. This decreased feeding was greatest in actinomycin D injected snails and least in the anisomycin group. Feeding behaviour had recovered in all three drug conditions by the 5 hr test to levels that of the saline and no injection control levels. These results are shown in Figure 8.21.

FIGURE 8.19

THE EFFECTS OF PSI ON FOOD CONSUMPTION

DOSE 1

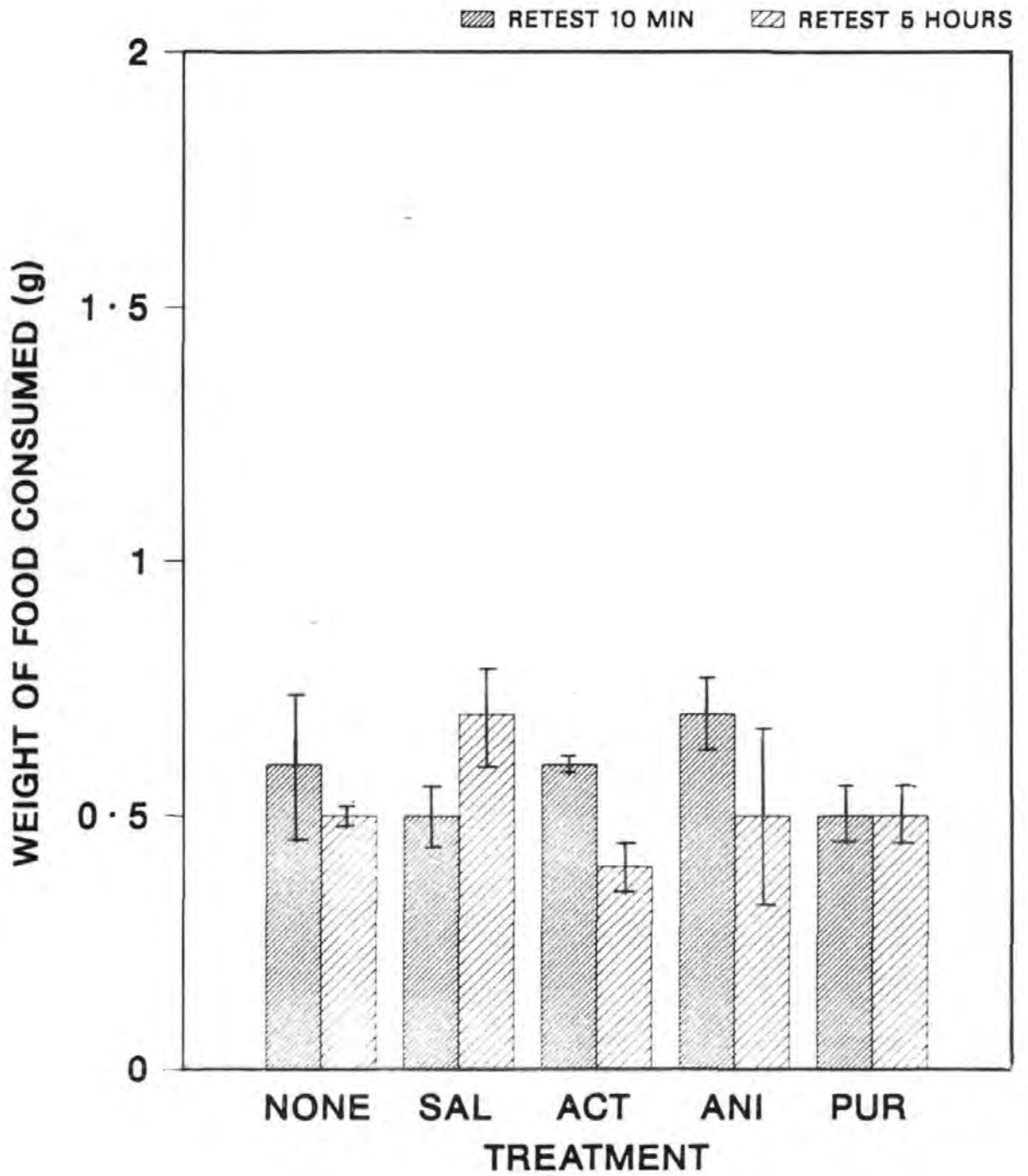


FIGURE 8.20

THE EFFECTS OF PSI ON FOOD CONSUMPTION  
DOSE 2

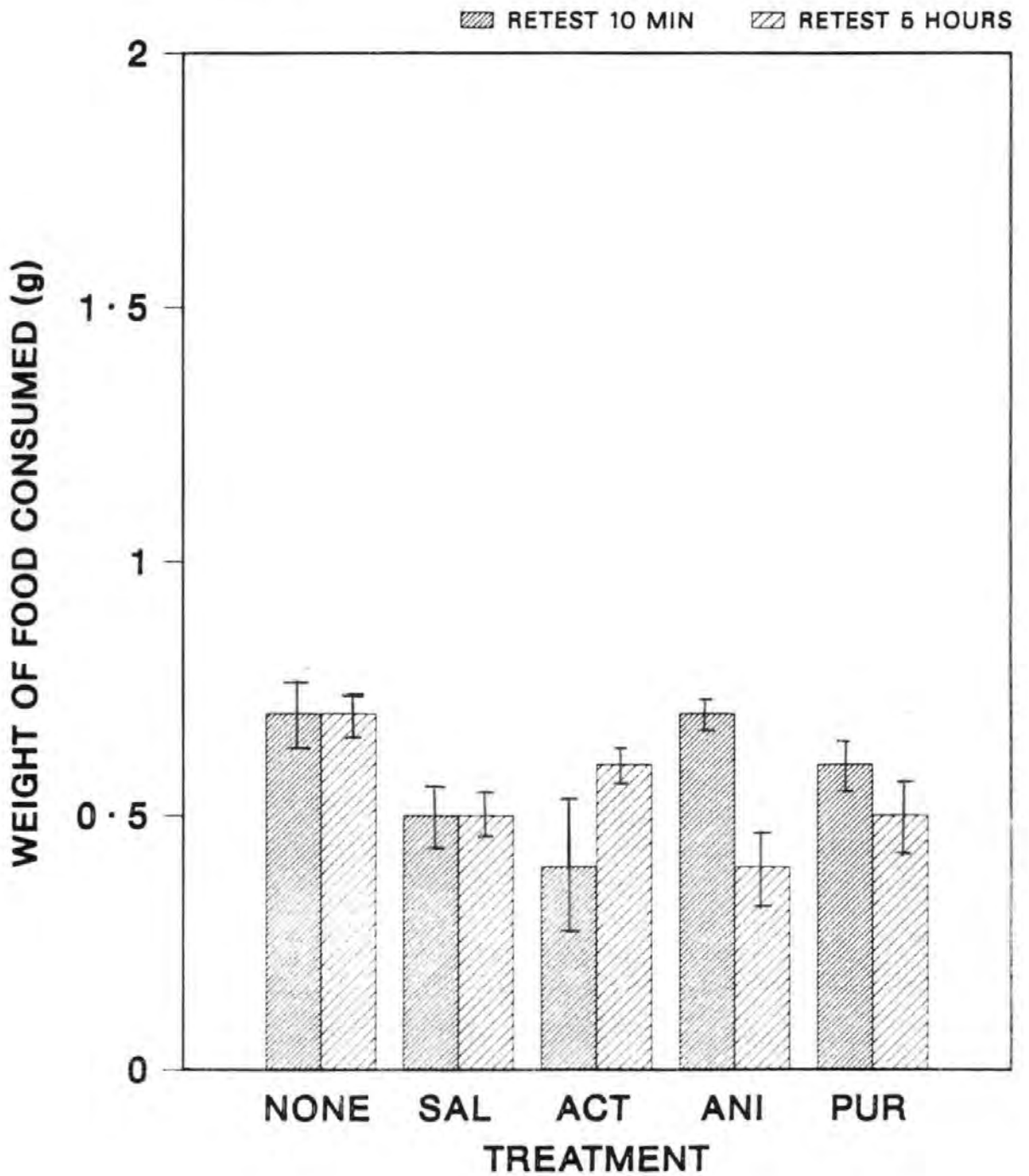
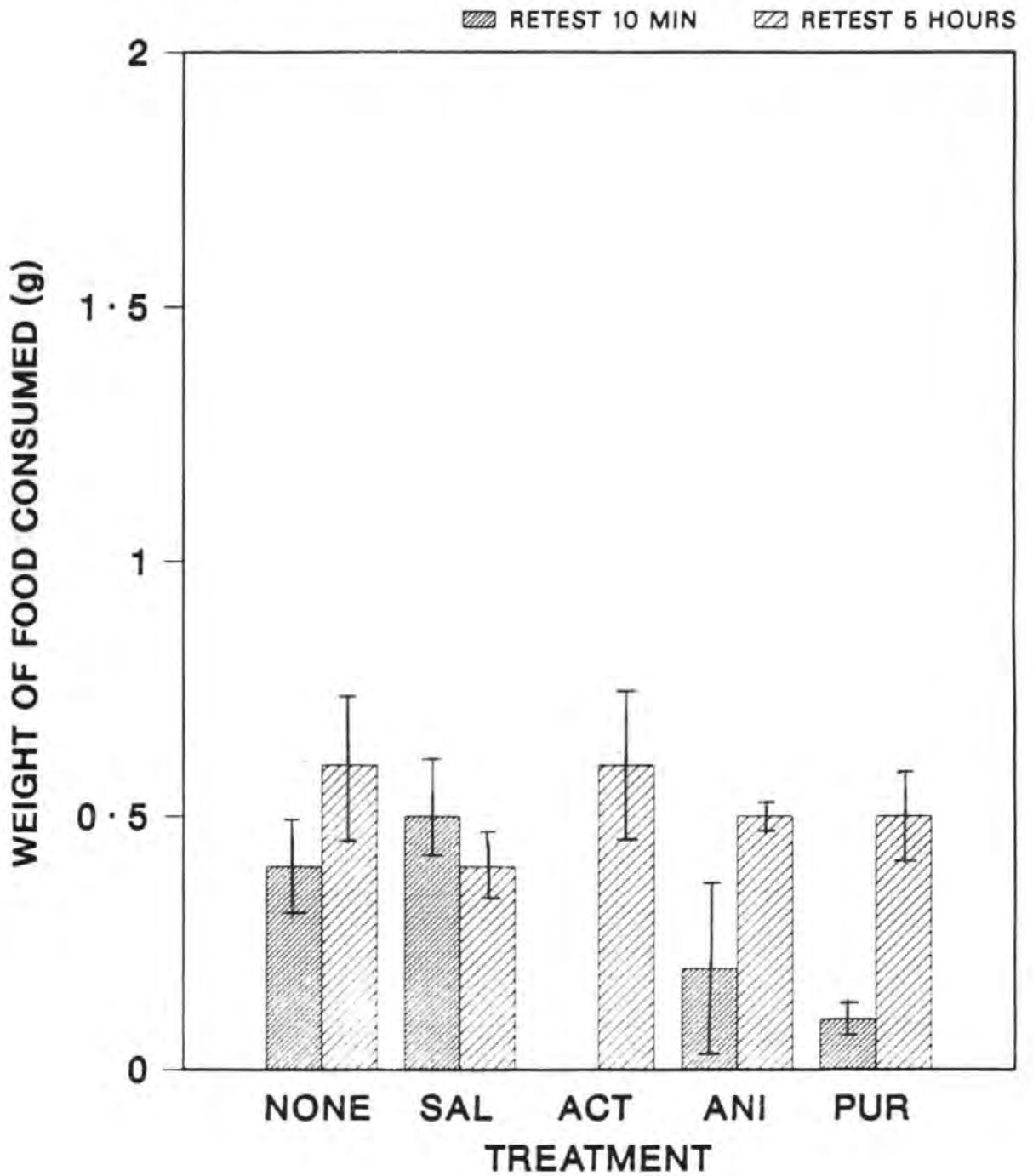


FIGURE 8.21

THE EFFECTS OF PSI ON FOOD CONSUMPTION  
DOSE 3



## CONCLUSION

From test 5, it was concluded that, at Dose 1 and 2, there was no effect of either anisomycin, actinomycin D, or puromycin evident at either test interval. Dose 3 did impair feeding behaviour immediately post injection. However, this had recovered by the 5 hrs test.

## EXPERIMENT 6

### AN INVESTIGATION OF THE EFFECTS OF THREE DOSES OF ANISOMYCIN, ACTINOMYCIN D AND PUROMYCIN ON THE RIGHTING REFLEX OF THE SNAIL

Twenty snails were assigned randomly to each of the three drug, saline, and no injection groups for each of the three doses of antibiotics. Half the animals were tested at 10 mins post-injection and the other half at 5 hrs post-injection. Duration taken to right itself was measured. The experiment was conducted on the nights of February 17, 18, 19, and 20, 1986, between the hours of 2200 and 1000 hrs. Temperature in the laboratory remained constant at 21 C.

## RESULTS

Analysis of these results in a 3 way ANOVA with between subject factors of dose, drug, and retest showed significant effects of Dose ( $F(2/270) = 811.620, p < .001$ ) and Drug

( $F(4/270) = 458.686, p < .001$ ). There was no significant effect of retest ( $F(1/270) < 1$ ), but there was a significant interaction of Dose x Drug ( $F(8/270) = 461.461, p < .001$ ). All other interaction failed to reach significance. ( $p > .5$ ).

#### DOSE 1

There was no effect on the time taken to complete righting behaviour in any of the three drug conditions at either post injection tests when compared to saline or no injection groups (see Figure 8.22).

#### DOSE 2

Again, there was no effect evident in any of the drug conditions at either post injection interval sampled with no difference in time taken to complete righting behaviour between any of the conditions at either post injection times sampled (see Figure 8.23).

#### DOSE 3

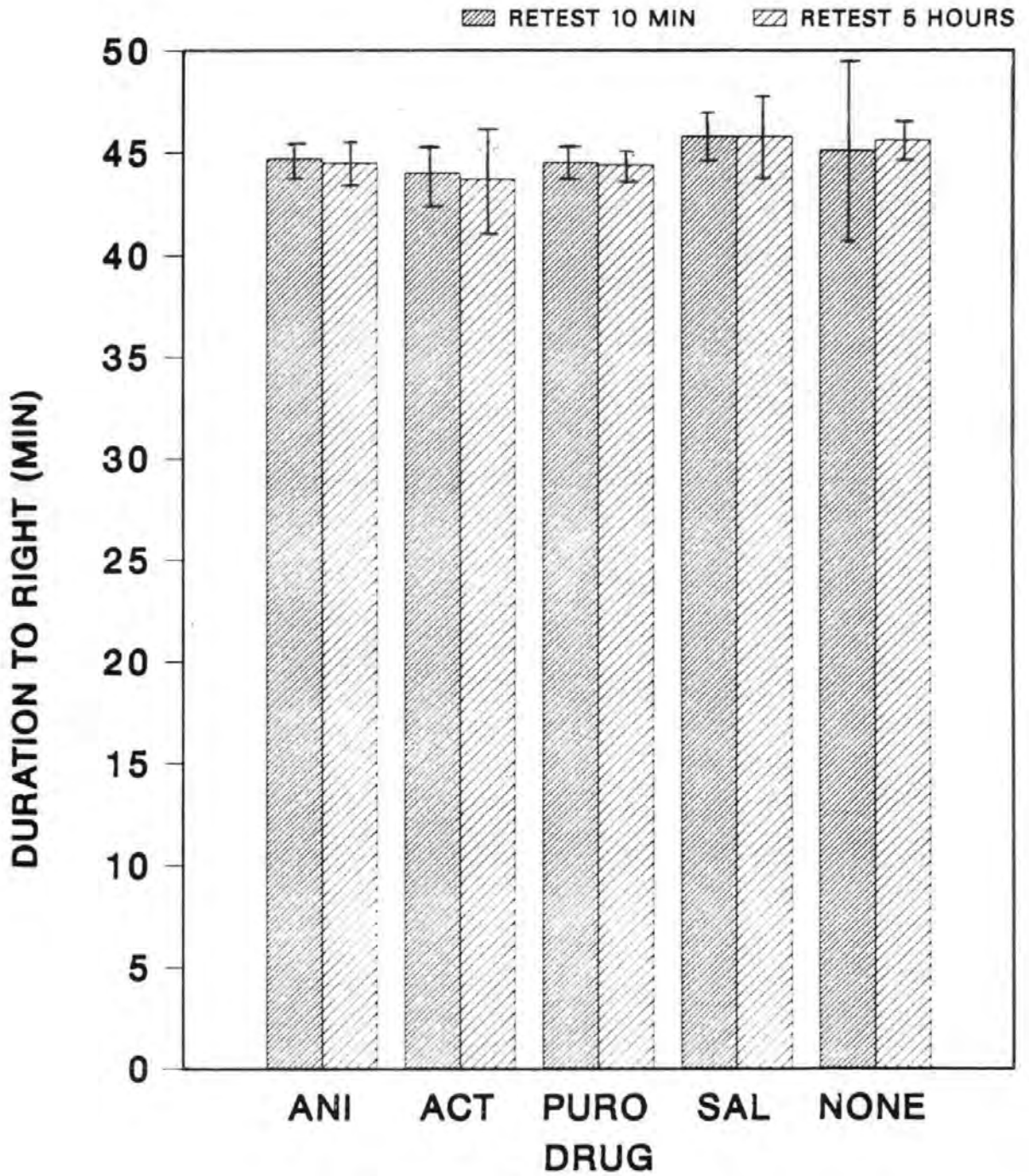
Snails in the anisomycin and actinomycin D conditions screened 10 mins post injection showed much longer durations to right themselves than the saline or no injection groups. Of the 10 snails in the puromycin group, all emerged from there shells but failed to right themselves. In the 5 hrs post injection test, anisomycin and actinomycin D treated snails still took longer to right than the control snails,



FIGURE 8.22

DRUG EFFECTS AT DOSE 1 ON RIGHTING RESPONSE

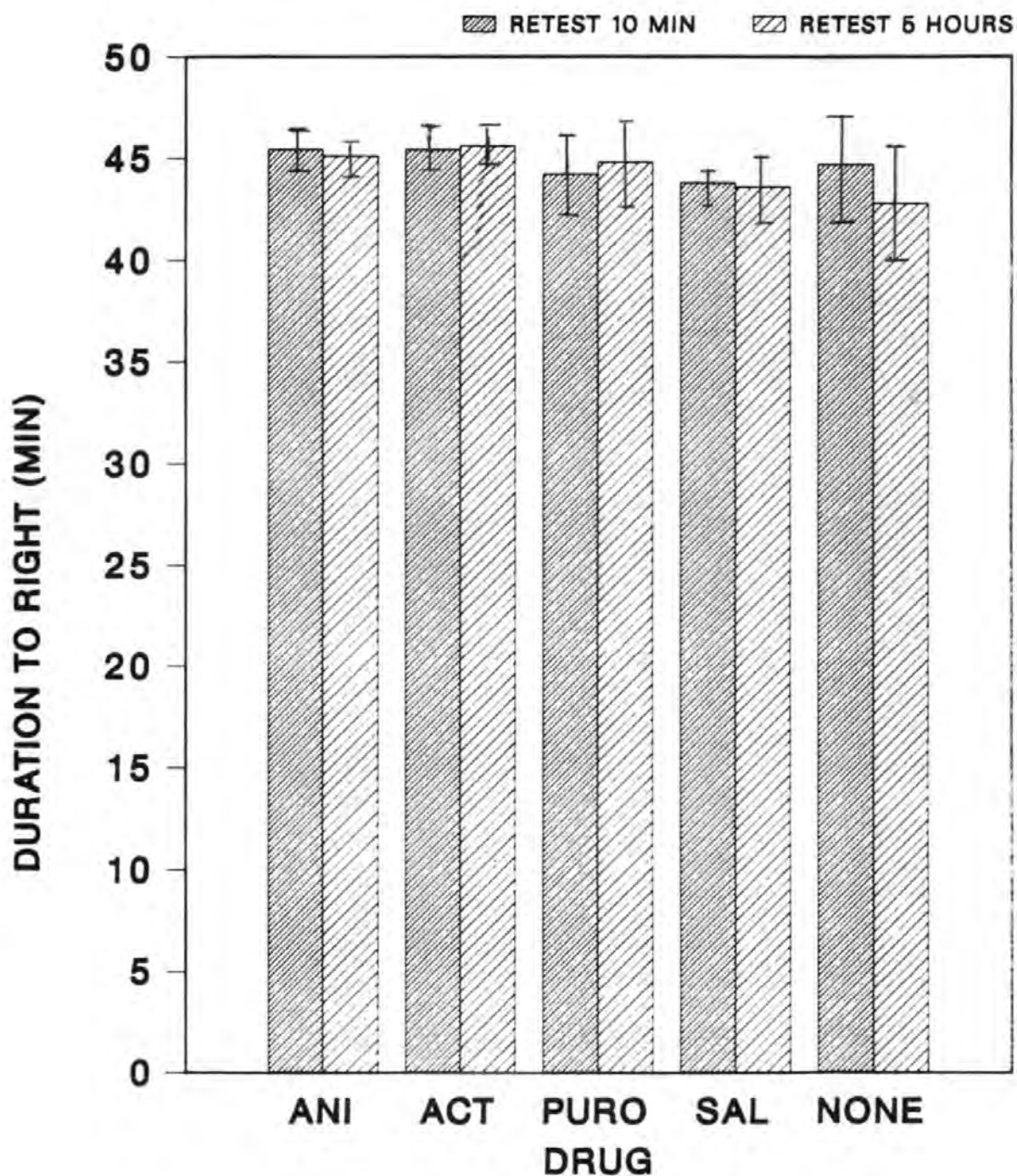
0.00025 mg/ml



**FIGURE 8.23**

**DRUG EFFECTS AT DOSE 2 ON RIGHTING RESPONSE**

0.025 mg/ml



but they righted quicker than the snails in the equivalent groups sampled 10 mins post injection. The puromycin injected snails still failed to right themselves in this test (see Figure 8.24).

## CONCLUSIONS

Anisomycin, actinomycin D and puromycin did not affect the motor and sensory coordination necessary to perform righting behaviour at Dose levels 1 and 2. However, at Dose 3 all drugs impaired the righting reflex, and this impairment only slightly recovered in the anisomycin and actinomycin D groups. Puromycin completely prevented righting at this dose.

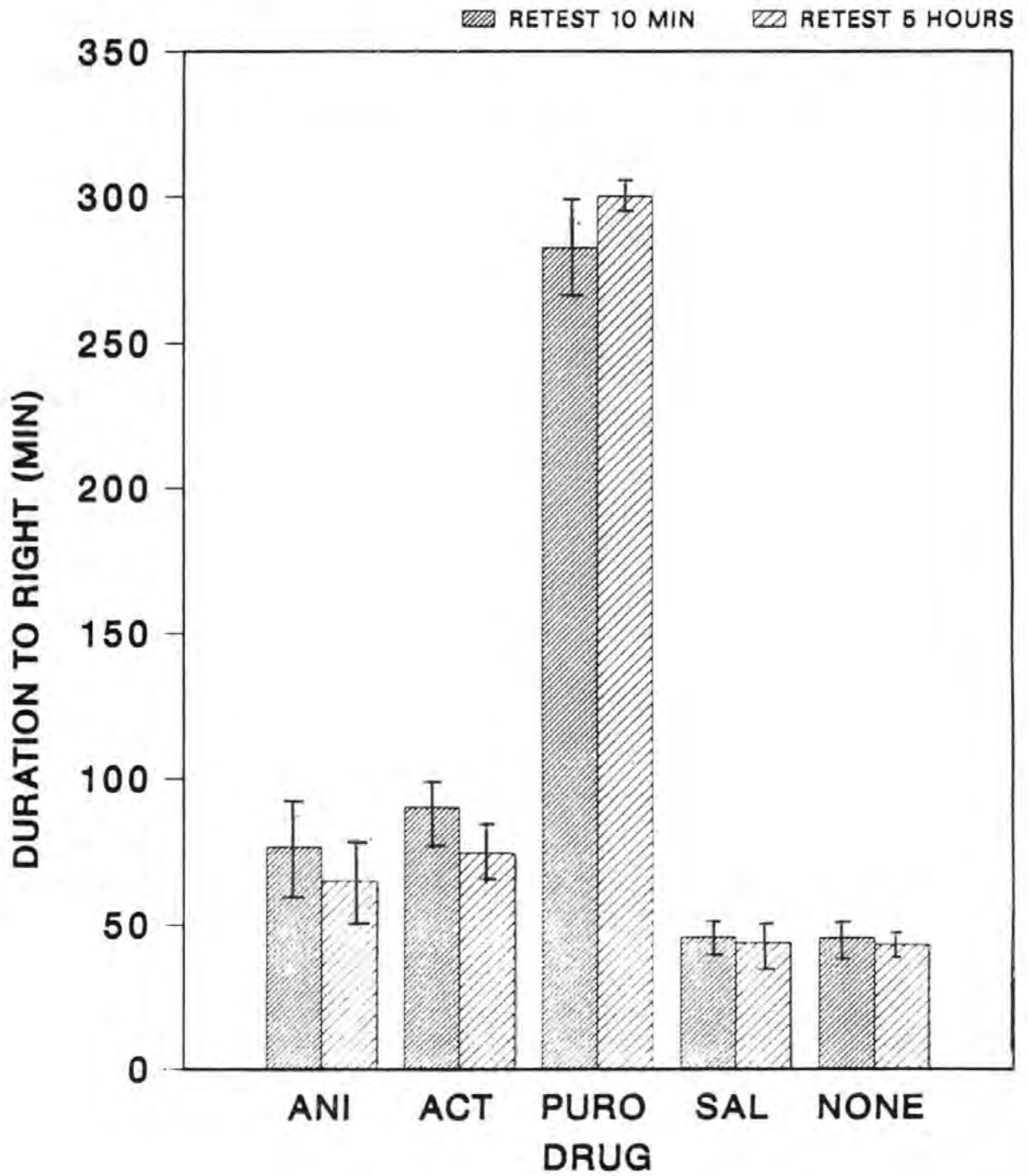
## SUMMARY OF FINDINGS FROM THE APPLICATION OF THE TEST BATTERY

The above battery of tests proved extremely useful in detecting debilitating effects of antibiotics at different dose levels. It also served to evaluate the injection method in terms of any trauma effects that injection may have had. It is concluded from this series of experiments that anisomycin, actinomycin D, and puromycin are suitable for an investigation of the effects antibiotics have on short and long term habituation in the snail at Dose 2 or lower (see Chapter 7, Table 1), as they have now been shown to have no detectable debilitating effects on various aspects of snail behaviour. Dose 3 proved to be unsuitable for further

FIGURE 8.24

DRUG EFFECTS AT DOSE 3 ON RIGHTING RESPONSE

2.5 mg/ml



NB. NOTE CHANGE IN SCALE

learning studies as it had <sup>debilitating</sup> effects on general behaviour. Cycloheximide proved to be totally unsuitable in any dose level for an investigation of learning. Optimal volume of injection was 0.1 ml; 0.3 ml was found to debilitate the snails.

The test battery reported above now provides us with more quantifiable evidence of antibiotic side effects in the snail and facilitates a deliniation of specific learning effects and general debilitation of the animal. The introduction of a no injection control group into the test battery design has shown that the injection methodology itself does not interfere with normal snail functioning. As a consequence of the above findings, the battery of tests developed could provide a useful and animal-economic facility for the screening of other pharmacological or immunological compounds that could be of use in the illucidation of a biological mechanism of learning and memory. Similar batteries of tests could be developed for other animal systems. The research reported in this chapter has established doses of three protein synthesis inhibitors that are free of any obvious side effects. These drugs, at these doses, were now utilised in a behavioural investigation of their effects on the short and long term habituation of the antennae withdrawal response.

## CHAPTER 9

### DOSE/EFFECT ANALYSIS OF THE ANTIBIOTICS ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN ON SHORT AND LONG TERM HABITUATION IN THE SNAIL HELIX ASPERSA

The plethora of research on the effects of protein synthesis on learning lacks conviction partly because many results cannot be generalised between laboratories, or, indeed, in some cases, be generalised within laboratories because of the variety of doses of drug employed. Researchers have tended to report a behavioural effect of a variety of antibiotic agents with only one dose of a given drug or a variety of doses within a narrow range. It is important in any pharmacological manipulation of an observable behaviour to establish adequate dose/effect relationships.

Gold et al. (1973) have suggested that dose of drug plays a vital role in the amnesic effects of antibiotics. Unfortunately, the available data for antibiotics is inadequate for a full investigation of the dose/effect relationship. Research in this area often shows different drugs to have different uptake levels necessitating pharmacological comparisons of behavioural effects between drugs at vastly different doses: e.g., Dunn et al., (1977) who compared amnesic effects of anisomycin (25 mg/kg), cycloheximide (120 mg/kg) and emetine (30 mg/kg). Studies of PSIs in intact invertebrates, though much less numerous, do report some dose/effect differences between drugs but these

are less marked than in vertebrates (e.g. Kerkut et al., 1973). Goelet et al. (1986) report that amnesic effects are found with doses of anisomycin (10 yg), emetin (100 yg), actinomycin D (50 yg/ml), and alpha-amatine (2 yg/ml). Unfortunately, these authors did not report dose/effect data which was adequate enough for inter-drug comparisons to be useful.

Dose of antibiotic has normally been expressed as milligrammes (mg) or microgrammes (yg) per kg of animal weight. This paradigm can lead to animals in a common condition receiving different volumes of drug. In light of the findings of the test battery (see Chapter 8), a standard volume/standard animal weight approach was adopted for the snails. This was facilitated in that samples of snails of required body weight could easily be collected from the wild or selected from the laboratory population.

The purpose of a dose/effect experiment is to measure the effect of various doses of a drug with a known biochemical effect on a measurable piece of behaviour or response. In this case, the effects of anisomycin, actinomycin D, and puromycin at different doses investigated in the previous two chapters are compared for their effects on both short and long term habituation of the dorsal antennae withdrawal response.

#### METHOD

**Subjects.** Two hundred and ten mature snails of weight (8g) were selected from a laboratory bred population. All

snails were laboratory housed in a 1 x 1 m glass vivarium which contained damp soil and turf.

Procedure. Each snail was in turn removed from the home vivarium. While the snail was enclosed in its shell it received a pedal injection of antibiotic or saline at a given dose. Five doses of each of three antibiotics found to be safe in the preceding chapter were compared with the effects of saline and no injection on short and long term habituation. The five doses employed were 0.025 yg/snail, 0.25 yg/snail, 2.5 yg/snail, 25 yg/snail, and 250 yg/snail. Snails in the saline group received a 0.1 ml injection of physiological saline. After the injection, each snail was then numbered and replaced in the home vivarium where the drug was given an incubation period of 30 mins. The snail was then relocated and placed on the experimental board (test board, apparatus orientation period, procedure for habituation, and stimulus were as reported in Experiment 1, Chapter 3). Trials required to reach criterion of habituation were recorded for this initial training session. The animal was then returned to the home vivarium for 5 hrs. It was then relocated and, after a second apparatus orientation period, retested for retention (retrials to habituation). Seventy snails were randomly allocated to each drug condition, within each drug condition, 10 snails received no injection, 10 snails received an injection of saline. Of the remaining snails, 10 were allocated to each of the 5 dose groups. All data collection was conducted between 0030 and 1130 Hrs on the mornings of May 6, 7, and 8 1986 with a laboratory temperature of 20 C.



## RESULTS

Results in terms of trials to habituation were analysed in a 3-way ANOVA with a within subject factor of trial (training/retest) and between subject factors of drug and dose. This revealed significant effects of Dose ( $F(6/189) = 82.396, p < .001$ ) and Trial ( $F(1/189) = 921.380, p < .001$ ) with a significant Dose x Trial ( $F(6/189) = 113.000, p < .001$ ) interaction. The differences between drugs failed to reach significance ( $F(2/189) < 1$ ), as did all other interactions.

Analysis of simple main effects revealed no difference between the five doses of antibiotic, saline and no injection groups in the initial training for all three antibiotics, ( $F's(1/189) < 1$ ). Thus, there was no effect on short term habituation at any dose for any of the three antibiotics. Analysis of retest data revealed a significant difference of dose ( $F(1/189) = 627.89, p < .001$ ). With Doses 1 and 2, there was no effect of any of the three drugs on long term habituation sampled at 5 hrs post training. Retention was evident in these groups as shown by significantly smaller number of trials to rehabituation than training trials ( $F's(1/189) = 453.01, 399.73, \text{ and } 324.96, p < .001$ ). Similar, retention was also found in the saline and no injection groups ( $F's(1/189) = 443.98 \text{ and } 401.12, p < .001$ ). Anisomycin, actinomycin D, and puromycin were all found to prevent long term habituation at Doses 3, 4 and 5 (all  $F's(1/189) < 1$ ). These effects are illustrated in Figures 9.1, 9.2, and 9.3.

FIGURE 9.1

DOSE EFFECTS OF ANISOMYCIN ON TRIALS TO HABITUATION IN TRAINING AND RETEST

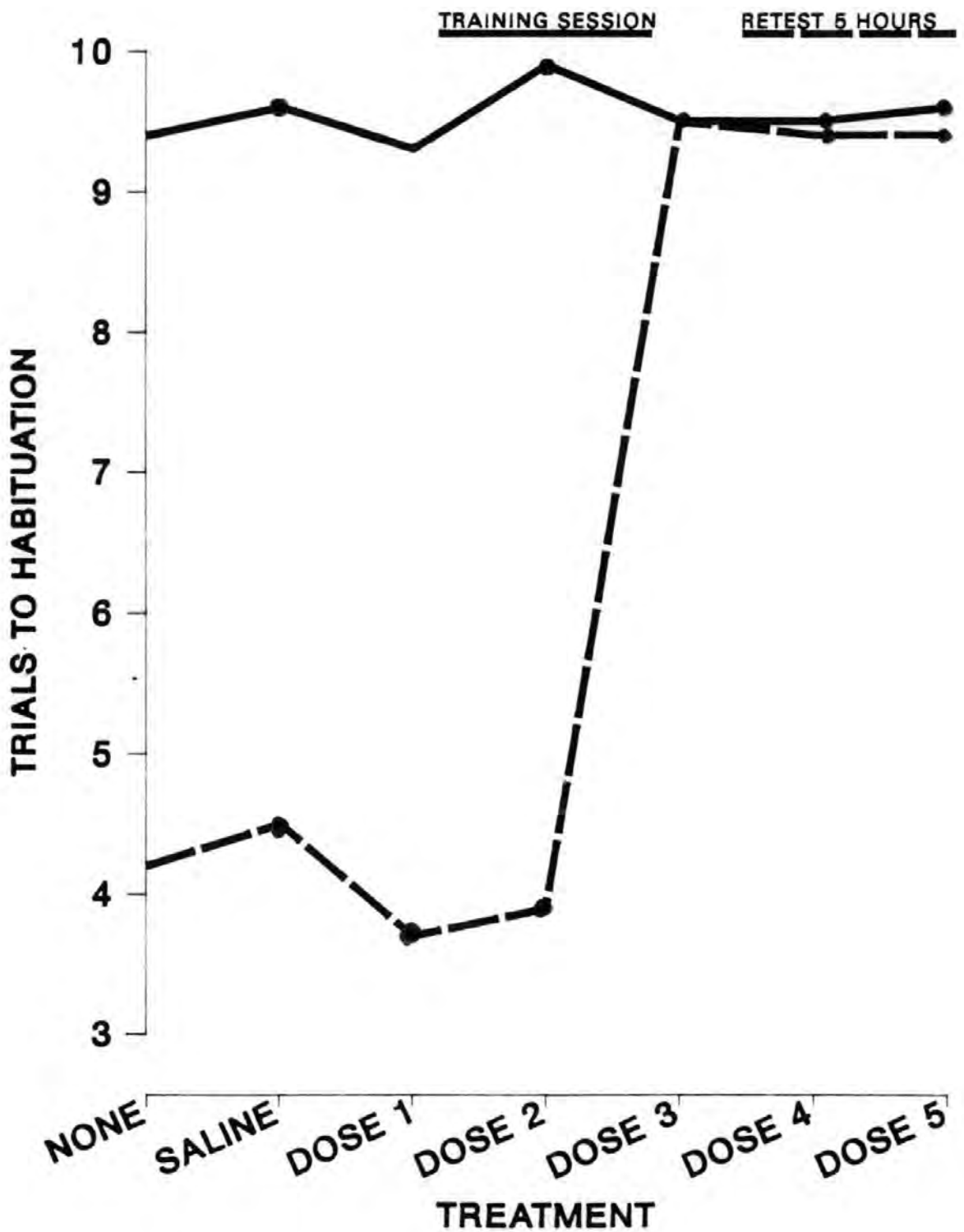


FIGURE 9.2

DOSE EFFECTS OF ACTINOMYCIN D ON TRIALS TO HABITUATION IN TRAINING AND RETEST

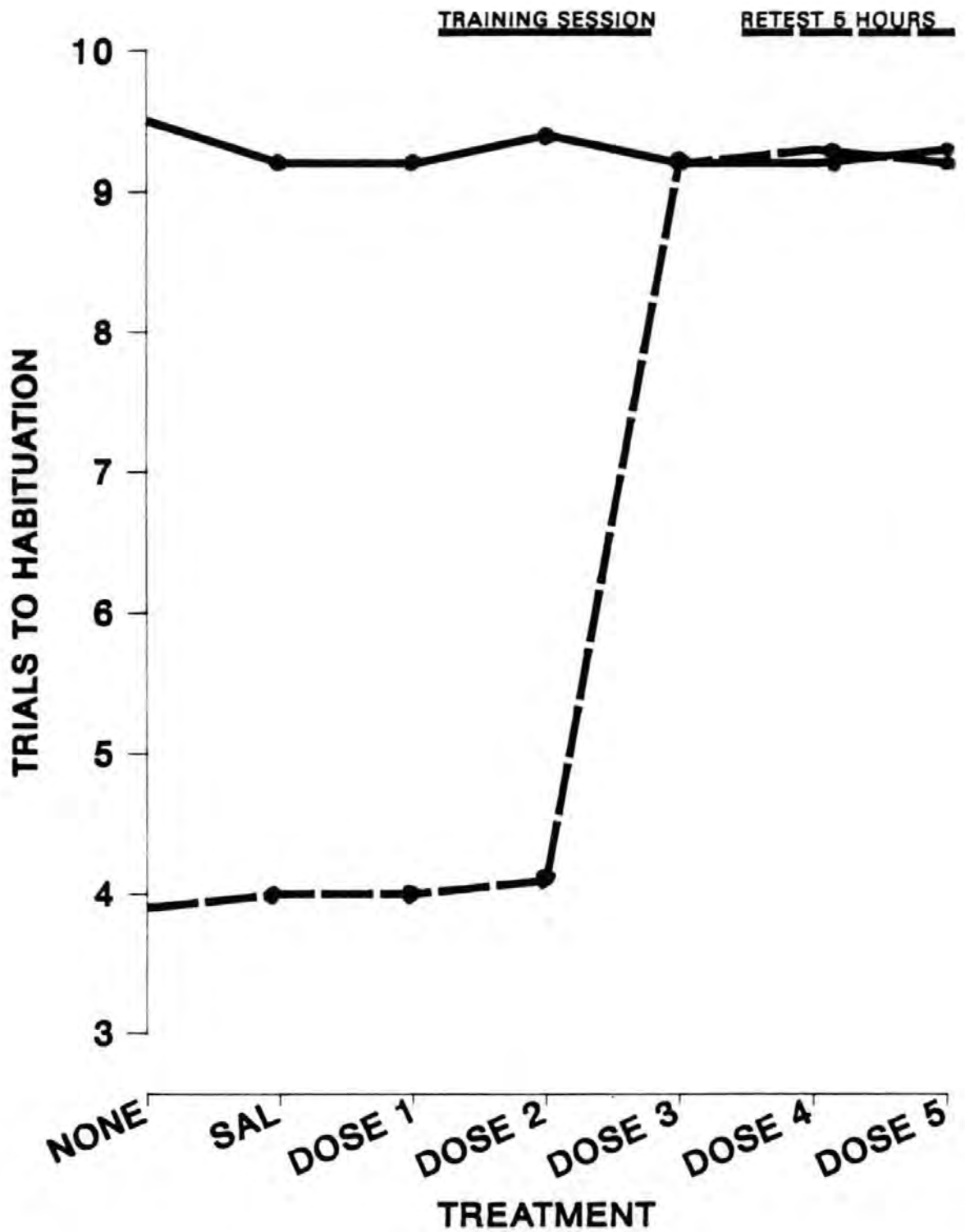
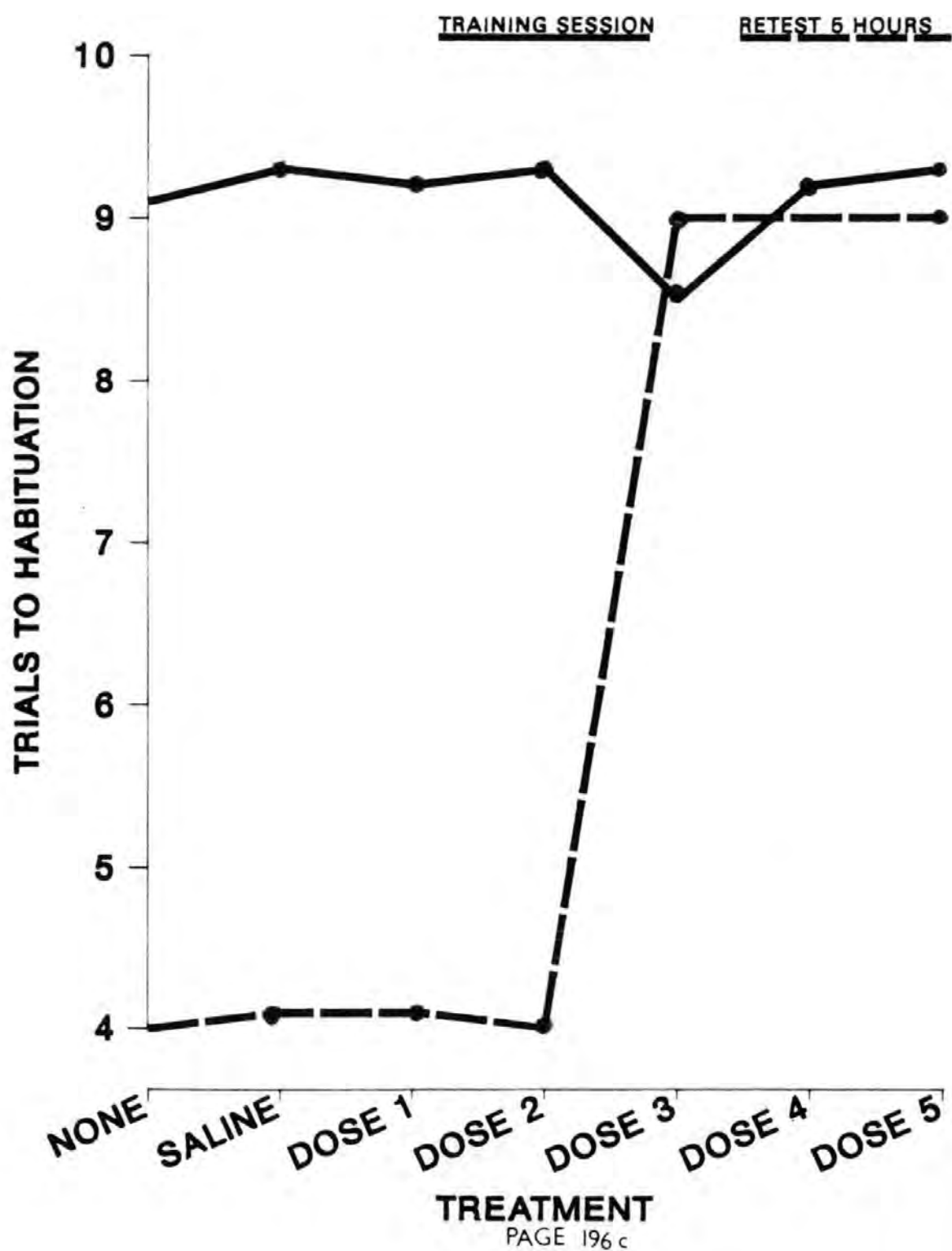


FIGURE 9.3

DOSE EFFECTS OF ANISOMYCIN ON TRIALS TO HABITUATION IN TRAINING AND RETEST



## DISCUSSION

The protein anti-metabolites anisomycin, actinomycin D, and puromycin had no effect on short term habituation at any dose compared to saline and no injection controls. However, these antibiotics disrupted long term retention at Doses 3 and all higher doses, and there was no difference between drugs in this effect. Normal long term habituation was evidenced with all three antibiotics at Doses 1 and 2. The above results suggest there is an effect of PSIs on long term habituation and that such an effect is dependent on the dose of drug employed. There was no effect in short term habituation at any dose.

It is interesting that, in this dose/effect analysis, there was no significant difference between the three antibiotics employed in their main effects on the learning. This apparent similarity despite different modes of action warrants further investigation of dose effects. While these results are in accord with biochemical evidence reported in Chapter 7, they show a similarity across drugs not previously reported in other animals.

The main effect that protein synthesis inhibition has no effect on short term habituation but can disrupt long term retention was further investigated as a function of dose in a series of three experiments reported in the next chapter.

## CHAPTER 10

### THE EFFECTS OF PSIs ON SHORT AND LONG TERM HABITUATION OF THE ANTENNAE WITHDRAWAL RESPONSE

Preliminary investigations reported in Chapters 7, 8, and 9 have established the inhibitory effects of anisomycin, actinomycin D, and puromycin and determined 'safe' side effect free doses of these drugs. Research reported in the previous chapter has indicated that these drugs to have no effect on short term habituation but at doses greater than 2.5 yg/snail they can prevent long term habituation.

Although these preliminary results are in accord with the literature, alternative explanations other than inhibition of protein synthesis, have been put forward to explain such results. The most common of these explanations of the apparent long term amnesia found with PSIs have included state dependency effects (where animals which have received PSI in training are tested without PSI at retest, a deficit in retention could be attributable to state dependent learning), short or long term drug induced performance debilitations, and drugs having short or long latency effects. Such alternative explanations have been encouraged by inadequate controls in PSI experiments for these effects.

A significant advance in this area of research would be the inclusion of adequate controls for these phenomena. Consequently, in this chapter, a series of three experiments investigating the effects of three PSIs researched in the

previous chapters commences a programme of research designed to further establish the main effects of PSIs on short and long term habituation, and to control for alternative explanations of these effects. In employing such control groups, the design facilitates analysis of drug effects at training, after training, and also at retest in order to see whether protein synthesis inhibition disrupts long term habituation at the encoding stage or at the retrieval stage.

Research in this chapter further investigates the dose/effect relationship found in Chapter 9. Dose effect studies have traditionally only looked for the relationship of dose of drug with performance on a given task, the possibility that different dose effects may interact with such phenomena as state dependent learning, or short and long term performance effects has largely been ignored.

## EXPERIMENT 1

### THE EFFECTS OF PUROMYCIN AT 3 DOSES ON SHORT AND LONG TERM HABITUATION OF THE THE DORSAL ANTENNAE WITHDRAWAL RESPONSE

Puromycin has been widely employed as a tool in the investigation of the biochemistry of learning (see Chapter 1). In order to establish its effect on short and long term habituation of the antennae withdrawal response, a study was conducted to investigate three doses of the drug found to be effective in the previous Chapter. A design was employed that controlled for short and long latency drug effects,

state dependent learning, and performance effects; all of which have been suggested as possible causes of the amnesic effects reported by other authors for these and other amnesic drug (Dunn, 1980; Nakajima, 1975). The experiment also allows analysis of drug effects at training, after training, and at retest to indicate whether protein synthesis inhibition disrupts long term habituation at the encoding stage or at the retrieval stage.

#### METHOD

Subjects & Dose. Four hundred and eighty snails were sampled in this study, in 16 experimental conditions (see Table 10.1) with 30 snails in each group. Of these 30 snails, 10 were allocated to each of three dose conditions: namely, Dose 1 (2.5 yg/snail), Dose 2 (25yg/snail), and 3 (250 yg/snail) all of which were seen to disrupt long term habituation in Chapter 9.

DESIGN. This design was utilised in all three experiments reported in this chapter. A sixteen condition experimental design was devised. These conditions are shown in Table 10.1. and explained below.



TABLE 10.1

TREATMENT PROTOCOLS FOR THE EXPERIMENTAL DESIGN

GROUP	TRAINING	REST 12 HOURS	RETEST
1	DRUG	DRUG	DRUG
2	DRUG	SALINE	DRUG
3	DRUG	SALINE	SALINE
4	DRUG	DRUG	SALINE
5	SALINE	SALINE	SALINE
6	SALINE	DRUG	SALINE
7	SALINE	DRUG	DRUG
8	SALINE	SALINE	DRUG
	NO TRAINING	REST 12 HOURS	RETEST
9	DRUG	DRUG	DRUG
10	DRUG	SALINE	DRUG
11	DRUG	SALINE	SALINE
12	DRUG	DRUG	SALINE
13	SALINE	SALINE	SALINE
14	SALINE	DRUG	SALINE
15	SALINE	DRUG	DRUG
16	SALINE	SALINE	DRUG

The experiment was divided into three sections which were training, rest for 12 hrs, and a subsequent retest. The treatments indicated in Table 10.1 show when the drug was active with respect to these sessions.

A series of comparisons were made in order to evaluate amnesic and possible confounding<sup>g</sup> effects of the PSIs.

COMPARISONS FOR GROUPS 1 TO 8

1 Each animal in these groups received initial training. The initial comparison was between training scores (trials to habituation) for Groups 1 to 4 and Groups 5 to 8. This shows any effect of drug on short term habituation and any short term performance effect.

2 Within group comparisons between training and retest scores to determine which groups show retention.

3 Comparison of retest scores of Groups 5 and 8 with

Groups 1, 2, 3, 4, 6, and 7 show if protein synthesis inhibition in training or immediately after can disrupt long term habituation.

4 Comparisons between groups that received drug in retest only with group that received no drug in retest (Group 5 with Group 8) to determine if drug had a performance effect at retest.

5 Comparisons between groups which received the drug only during training (2 and 3) and those which received it only after training (Groups 6 and 7). This comparison gave some indication of the time of action of the drug.

6 Similarly, comparisons of retest scores between groups that that received drug in both training and immediately after training (Groups 1 and 4) with groups that received drug in training only, (Groups 2 and 3) and immediately after training only (Groups 6 and 7), show if multiple injection increased amnestic effects of the drug.

7 The design can also test for long term performance effects by comparisons between groups that received drug in retest (Groups 1, 2, 7, and 8) with those that did not (Groups 3, 4, 5, and 6), and supports this comparison by comparisons with no training conditions (groups 9-16).

8 Comparisons between group 2 and group 3, in terms of retest scores tests for drug dependent learning.

9 Comparisons of Group 5 with Group 8 retest scores further shows if the drugs have a short term performance effect at retest.

## COMPARISONS FOR GROUPS 9 to 16

The treatments of Groups 1 to 8 were duplicated in Groups 9 to 16 except that in these groups snails received no initial training.

10 Thus, comparisons between indentical treatment groups (e.g Group 1 with Group 9) further shows if the drug had any long term performance effects.

11 Comparisons between retest scores of Groups 5 and 8 with Groups 13 and 16 shows retention of training over the twelve hour retest.

12 Comparisons between groups which had had the drug during or just after the no training period (9 to 12, 14 and 15) with group 13 which had had no drug at all shows if the drug has any long latency effects on performance.

13 Comparisons between the groups which had the drug during the retest period (16) and the drug free group (13) further shows any short term effects on learning.

14 Comparison between the Group which had the drug at each possible point (9) with the drug free group (13) shows any cummulative effects of the drug on performance.

### PROCEDURE

Training and retest sessions consisted of repeated presentations of the tactile stimulus (aluminium prod) with an ISI of 30 secs until the criterion of habituation was acheived. Short term habituation was measured as trials required to habituation and long term habituation was

measured in terms of savings in trials to habituation at retest (12 hrs post-training).

Snails in Groups 1 to 8 all received an initial training session followed by a 12 hr rest period spent in the home vivarium. The animal was relocated at the end of this procedure from its identification number, and then received the retest. Snails in Groups 9 to 16 were treated in an identical way with the exception that these animals received no initial training. However, they were still handled and placed on the test apparatus for an equivalent period of time. Substitute snails were trained in each group to replace snails that did not emerge from their shells at retest.

Injection procedure was as reported in Chapter 7. Pretraining injections were administered 30 minutes prior to commencement of training, rest period injections were given immediately after training, and retest injections were given 30 minutes prior to retest session.

A blind procedure was used. All snails were numbered, and at retest, they were located by an assistant who masked their numbers. All injections were from pre numbered masked syringes following a number code to ensure the experimenter was unaware which dose he was injecting. The experiment was conducted on the nights of May 17, 18, 19, 20, and 21, 1986, between the hours of 1600 and 1100. Temperature in the laboratory varied from 17 to 21 C.

## RESULTS

TABLE 10.2

MEAN TRIALS TO HABITUATION IN TRAINING AND  
RETEST FOR EACH GROUP AT 3 EXPERIMENTAL DOSES

GROUP	TRAINING			RETEST		
	DOSE 3	DOSE 4	DOSE 5	DOSE 3	DOSE 4	DOSE 5
1	10.5	10.0	10.3	10.1	10.2	10.4
2	10.7	10.9	10.5	10.5	10.6	10.3
3	10.0	10.8	10.6	9.9	10.8	10.5
4	10.9	10.2	10.7	10.9	10.0	10.0
5	10.3	10.8	10.9	4.1	3.9	4.6
6	10.5	10.1	10.4	10.7	10.0	11.1
7	10.2	10.8	10.8	10.8	10.7	10.9
8	10.8	11.2	10.0	4.0	4.2	4.1
9	-	-	-	10.9	10.0	10.8
10	-	-	-	11.0	10.5	10.0
11	-	-	-	10.3	10.4	10.3
12	-	-	-	9.9	10.4	10.8
13	-	-	-	10.9	11.0	10.0
14	-	-	-	10.0	10.1	10.0
15	-	-	-	10.1	10.9	10.2
16	-	-	-	10.6	10.4	10.8

Trials required to habituation in retest were analysed in a 2-way ANOVA with between subject factors of dose and group and revealed a significant effect of group ( $F(15/432) = 78.067, p < .001$ ) and no effect of dose ( $F(2/432) < 1$ ). The two way interaction failed to reach significance ( $p > .5$ ).

A further 3 way ANOVA with a within subject factor of test (training/retest) and between subject factors of group and dose was conducted to compare trials to criterion in training with those in retest for Groups 1..8 who had received training this showed a significant effect of test ( $F(1/432) = 142.119, p < .001$ ) and group ( $F(7/432) = 73.746, p < .001$ ) with a significant Test x group interaction ( $F(7/432) = 73.237, p < .001$ ). All other interactions failed to reach significance ( $p > .5$ ). Again, there was no effect of

dose ( $F(2/432) < 1$ ).

The comparisons described in the method section were then carried out. This revealed that with regards to short term habituation, Groups 1 to 4 showed no effect of drug in training compared to saline injected snails (Groups 5 to 8) ( $F's(7/432) <$ ). It was concluded from this that puromycin had no effect on short term habituation. The same comparison helped rule out any short term latency effects of the drug. Further evidence of no short term effects of drug was provided by comparing Groups 16 and 13 ( $F(7/432) < 1$ ).

Within group comparisons revealed significant retention in Groups which received no drug in either training or immediately after training, Group 5 ( $F(7/432) = 327.00$ ,  $p < .001$ ), and Group 8 ( $F(7/432) = 299.87$ ,  $p < .001$ ).

Comparisons between retest scores of Group 5 and 8 with Groups 13 and 16 showed retention of training over 12 hrs ( $F(7/432) = 718.64$ ,  $p < .001$ ).

Further analysis of retest scores revealed that retest scores in Groups 5 and 8 which received no drug in training or immediately after training required fewer trials to habituation than snails that had received, first, drug in training (Groups 2, and 3) ( $F(7/432) = 619.82$ ,  $p < .001$ ), second, drug immediately after training (Group 6 and 7), (this comparison also serves to negate an explanation of the long term effects as state dependent learning) ( $F(7/432) = 491.90$ ,  $p < .001$ ), and third, snails that had received drug in training and immediately after training (Group 1, and 4) ( $F(7/432) = 357.49$ ,  $p < .001$ ). Similarly, snails that received drug in retest only (Group 8) showed no effect on retest

score compared to Group 5 which received no drug in any test ( $F(7/432) < 1$ ). This showed the drug was not having a performance effect at retest. Comparisons of retest scores between snails that had received drug in both training and immediately after training (Group 1 and 4) with snails that received drug in training only, showed no significant difference  $F(7/432) < 1$ ). This was also the case when Groups 1 and 4 were compared with snails that received the drug immediately after training ( $F(7/432) < 1$ ). Thus there was no greater amnesia from multiple injections than evidenced by inhibition during training only or after training only. Inhibition of protein synthesis after this period had no effect on long term habituation (see Group 5 compared with Group 8). Further, Comparison 14 (Groups 9 with 13) showed that multiple injections did not have a cumulative effect on performance ( $F(7/432) < 1$ ). Comparisons between snails that received drug in retest (Groups 1, 2, 7, and 8) with those that did not (Groups 3, 4, 5, and 6) showed the drug did not have a performance effect at retest ( $F(7/432) < 1$ ).

Comparisons were also made between trained groups and non trained groups that had received the same injection protocols to demonstrate that the drugs are not having a long term latency effect. The eight training groups were each compared to the no training group with same injection protocol. There were no significant differences between retest scores in any comparison ( $F$ 's(7/432)  $< 1$ ) except Group 5 with 14 ( $F(7/432) = 541.63, p < .001$ ), and Group 8 with 16 ( $F(7/432) = 497.99, p < .001$ ). This conclusion was further supported by Comparison 11, with Groups 9 to 12, 14 and 15

with Group 13 ( $F_{(7/432)} < 1$ ).

The amnesic effects of injections during training evidenced at retest cannot be attributed to state dependency as disruption of long term habituation was also evident from the immediately post training injection groups as well (Group 6 and 7). State dependency effects are further negated by the comparison of retest scores from Groups 1 and 2 with Groups 3 and 4 ( $F_{(7/432)} < 1$ ), Groups 2 with Group 3 ( $F_{(7/432)} < 1$ ), also Groups 5 and 6 with Groups 7 and 8 ( $F_{(7/432)} < 1$ ).

### DISCUSSION

The results of Experiment 1 show that puromycin at all tested doses (2.5 yg/snail, 25 yg/snail, & 250 yg/snail) had no effect on short term habituation of the dorsal antennae withdrawal response, but, when injected either before or shortly after training, puromycin was capable of total disruption of long term habituation. It was also found that with all three doses there was no effect of the drug administered immediately before retest. These results cannot be explained by state dependent learning, short or long term performance effects, or short or long term drug latency effects at any of the three doses used.



## EXPERIMENT 2

### THE EFFECTS OF ACTINOMYCIN D AT 3 DOSES ON SHORT AND LONG TERM HABITUATION OF THE DORSAL ANTENNAE WITHDRAWAL RESPONSE

Actinomycin D has not been as widely used in investigations of the biochemical basis of learning and memory (see Chapter 1). It disrupts protein synthesis by inhibiting RNA synthesis. Thus, information from the DNA is not properly transcribed to mRNA which results in inhibited protein synthesis (see Chapter 7).

Actinomycin D at the same three doses found to have an effect in Chapter 9 were used in the same Experimental design reported in Experiment 1.

#### METHOD

Subjects. Subjects were 480 mature Helix aspersa collected from the Stonehouse area of Plymouth on the night of May 22, 1986 and laboratory housed as reported in Experiment 1, Chapter 3. All subjects were numbered for identification and of standard weight.

Procedure. Dose of drug, experimental design, habituation procedure, stimulus, interstimulus interval, criterion of habituation and injection protocols were as reported in Experiment 1. All data collection was conducted between 1600 and 1130 on the nights of May 23, 24, 25, 27, 28 and 29, 1986. Laboratory temp. varied from 18 to 21 C.

## RESULTS

TABLE 10.3

MEAN TRIALS REQUIRED TO REACH HABITUATION IN TRAINING AND  
AND RETEST IN EACH TREATMENT PROTOCOL FOR THREE DOSES OF  
ACTINOMYCIN D

GROUP	TRAINING			RETEST		
	DOSE 1	DOSE 2	DOSE 3	DOSE 1	DOSE 2	DOSE 3
1	10.3	10.2	10.9	9.9	10.3	10.8
2	10.9	10.4	10.7	10.6	10.4	10.6
3	10.7	10.7	10.1	10.7	10.9	10.1
4	10.1	10.8	10.0	10.5	10.6	10.0
5	10.9	10.4	10.6	4.9	4.4	4.5
6	10.7	10.6	10.9	10.9	11.4	10.8
7	10.3	10.4	10.0	10.5	10.2	10.0
8	10.1	10.3	10.2	4.3	4.2	4.4
9	-	-	-	10.9	10.1	10.0
10	-	-	-	10.0	11.2	10.8
11	-	-	-	10.3	10.4	10.2
12	-	-	-	9.7	11.0	10.1
13	-	-	-	10.6	10.7	10.5
14	-	-	-	10.3	10.1	10.7
15	-	-	-	11.6	10.5	10.0
16	-	-	-	10.0	10.3	10.1

Trials required to reach criterion of habituation at retest were compared from all 16 groups in a 2-way ANOVA with between subject factors of dose and group. This showed a significant effect of group ( $F(15/432) = 80.826, p < .001$ ) but not of dose ( $F(2/432) = 0.670, p > .5$ ), as was the case with puromycin.

A 3-way ANOVA, with a within subject factor of test (training/retest) and between subject factors of group and dose, conducted on Groups 1..8 which had received both training and a retest revealed significant effects of test ( $F(1/432) = 157.764, p < .001$ ) and condition ( $F(7/432) = 72.993$  with a significant interaction of test x condition ( $F(7/432) = 77.668, p < .001$ )). All other interactions failed

to reach significance ( $p > .5$ ). Again, there was no effect of dose ( $F(2/432) < 1$ ).

Results were further analysed as reported in Experiment 1, using the comparisons outlined in the design section of Experiment 1. There was no effect of actinomycin D on short term habituation when training session trials to habituation were compared between drug in training groups and saline in training groups ( $F(7/432) < 1$ ). Thus, again, this comparison ruled out this drug as having a short term latency or performance effect. The comparison between Groups 16 and 13 also supported this ( $F(7/432) < 1$ ).

Within group comparisons revealed significant retention in groups which received no drug in either training or immediately after training, Group 5 ( $F(7/432) = 286.99$ ,  $p < .001$ ), and Group 8 ( $F(7/432) = 341.64$ ,  $p < .001$ ). Similarly, there was no difference between retest scores in these two groups despite the fact that Group 8 had received the drug in retest ( $F(7/432) < 1$ ). Thus actinomycin D was not having a performance effect in retest.

Comparisons between retest scores of snails in Groups 5 and 8 with snails in Groups 13 and 16 showed retention over 12 hrs ( $F(7/432) = 679.83$ ,  $p < .001$ ) when protein synthesis was intact during and after training.

Retest scores also revealed that group 5 and 8 required fewer trials to rehabituation than snails which received actinomycin D in training (Groups 2, and 3) ( $F(7/432) = 458.91$ ,  $p < .001$ ), immediately after training (Groups 6 and 7) ( $F(7/432) = 491.90$ ,  $p < .001$ ) (again this comparison negates an explanation of actinomycin D's effect

as state dependent learning), and snails that received actinomycin D in both training and immediately after training (groups 1 and 4) ( $F(7/432) = 291.72, p < .001$ ).

Comparisons between snails that had received the drug in both training and immediately after training (groups 1 and 4) with groups receiving drug in training only, showed no significant difference between retest scores ( $F(7/432) < 1$ ). This was also the case with groups which received drug immediately after initial training ( $F(7/432) < 1$ ). Thus with actinomycin D there was no greater amnesia with multiple injections of drug, and multiple injections produced no debilitation of the animals.

Comparisons between snails that received actinomycin D in retest (Groups 1, 2, 7, and 8) with those that did not (Groups 3, 4, 5, and 6) showed this PSI did not have a performance effect at retest ( $F(7/432) < 1$ ). Further, comparisons of scores between Groups 9 to 12, 14, and 15, with Group 13, also supported this ( $F(7/432) < 1$ ).

As in Experiment 1, comparisons were also made between trained groups and non trained groups which received the same injection protocols. These comparisons demonstrated that actinomycin D was not having a long term latency effect. The eight trained groups were each compared to the non trained groups with the same treatment protocol. There were no significant differences between retest scores in any comparison ( $F(7/432) < 1$ ) except the comparison between Group 5 and 14 ( $F(7/432) = 592.81, p < .001$ ), and group 8 with 16 ( $F(7/432) = 497.99, p < .001$ ). Similarly, Comparison 14 (Group 9 with 13) showed multiple injections did not have a

cumulative effect on performance ( $F(7/432) < 1$ ).

That amnesic effects of injections during training evidenced at retest cannot be attributed to state dependency as disruption of long term habituation was also evident from the post training injection groups (Groups 6 and 7). State dependency effects are further negated by the comparison of retest scores from Groups 1 and 2 with Groups 3 and 4 which proved non significant ( $F(7/432) < 1$ ), also Groups 5 and 6 with Groups 7 and 8 ( $F(7/432) < 1$ ). Also there was no difference between Group 2 or Group 3.

#### DISCUSSION

At all doses, there was no effect of actinomycin D on short term habituation. However, at all doses, there was no evidence of any long term retention of the learning if the drug inhibited protein synthesis during or immediately after training. Actinomycin D was found to have the same disruptive properties as puromycin at the same doses, and, again, these effects could not be attributed to state dependency or drug performance effects.

### EXPERIMENT 3

#### THE EFFECTS OF ANISOMYCIN AT 3 DOSES ON SHORT AND LONG TERM HABITUATION OF THE DORSAL ANTENNAE WITHDRAWAL RESPONSE

The above experiment was then replicated for the same three doses of the third, tested antibiotic; anisomycin. These doses were shown to be side effects free (see Chapter 8). Anisomycin is an antibiotic that was developed for the treatment of rectal tumors in children and because of its elegant stereochemistry has been widely used in the neurobiology of learning research (see Chapter 1). Its mode of action is similar to puromycin in that it disrupts the construction of the peptide chain at the ribosome (see Chapter 1).

#### METHOD

Subjects. Four hundred and eighty snails of standard weight, collected from the Stonehouse area of Plymouth were employed in this study. Laboratory housing was as reported in Experiment 1, Chapter 3.

Procedure. Doses, experimental control groups, habituation procedure, stimulus, interstimulus interval, criterion of habituation and injection protocols were as reported in Experiment 1. Experiment 3 was conducted between 2400 and 0845 on the mornings of June 1, 2, 3, 4 and 5, 1986. Temperature in the laboratory varied from 16 - 19 C.

## RESULTS

TABLE 10.4

MEAN NUMBER OF TRIALS TO HABITUATION REQUIRED IN SHORT AND LONG TERM HABITUATION IN EACH EXPERIMENTAL CONDITION

GROUP	TRAINING			RETEST		
	DOSE 1	DOSE 2	DOSE 3	DOSE 1	DOSE 2	DOSE 3
1	11.3	10.8	10.7	10.9	10.5	10.4
2	10.0	10.6	10.3	10.5	10.7	10.0
3	9.7	11.0	10.2	10.0	10.8	10.6
4	10.7	10.7	10.4	10.5	10.8	10.3
5	10.4	10.5	10.3	4.8	3.9	4.2
6	10.0	10.4	11.3	10.3	10.2	10.9
7	12.0	10.6	10.2	10.0	11.2	10.3
8	10.8	10.3	10.6	4.0	4.2	4.6
9	-	-	-	10.8	10.5	10.3
10	-	-	-	11.0	10.3	10.0
11	-	-	-	9.9	10.6	10.8
12	-	-	-	10.3	10.4	10.6
13	-	-	-	10.2	10.4	10.9
14	-	-	-	11.0	10.6	10.5
15	-	-	-	10.0	10.1	10.3
16	-	-	-	10.5	10.8	10.1

Trials required to reach criterion in retest session or its equivalent were analysed for all 16 conditions to compare trained groups with no training under the same drug regimes in a 2 way ANOVA with between subject factors of group and dose to reveal a significant effect of group ( $F(15/432) = 86.182, p < .001$ ). There was no effect of dose ( $F(2/432) < 1$ ) and no group x dose interaction ( $F(30/432) < 1$ ).

A further 3-way ANOVA with a within subject factor of test and between subject factors of group and dose was then conducted on trial to criterion in training and retest for groups which had received training (1...8) to measure long term retention. This showed a significant effect of group

( $F(7/432) = 82.056, p < .001$ ) and test ( $F(1/432) = 209.834, p < .001$ ) with a significant group x test interaction ( $F(7/432) = 79.111, p < .001$ ). There was again, no effect of Dose ( $F(2/432) < 1$ ). All other interactions failed to reach significance ( $p > .5$ ).

Further analysis by comparisons outlined in Experiment 1 revealed the same effects as reported in the above two experiments. With regards to short term habituation, Groups 1 to 4 showed no effect of drug in training compared to saline injected snails (Groups 5 to 8) ( $F(7/432) < 1$ ). It was concluded from this that anisomycin had no effect on short term habituation at any of the above doses. The same comparison negated any short term latency effects of the drug, and was supported by Comparison 13 (Group 16 with Group 13) ( $F(7/432) < 1$ ).

Within group comparisons revealed significant retention in groups that received no drug in either training or immediately after training, Group 5 ( $F(7/432) = 216.04, p < .001$ ), and Group 8, ( $F(7/432) = 381.45, p < .001$ ). In all other trained groups retention was inhibited ( $F(7/432) < 1$ ).

Comparisons between retest scores of Group 5 and 8 with Groups 13 and 16 showed retention of training over 12 hrs ( $F(7/432) = 698.57, p < .001$ ).

Retest score analysis also revealed retest scores in Groups 5 and 8 which received no drug in training or immediately after training required fewer trials to habituation than snails that had received anisomycin in training (Groups 2 and 3) ( $F(7/432) = 701.85, p < .001$ ), drug immediately after training (Group 6 and 7) ( $F(7/432) =$



453.49,  $p < .001$ ), and snails that have received drug in training and immediately after training (Group 1 and 4) ( $F(7/432) = 529.07$ ,  $p < .001$ ). Similarly, snails that received drug in retest only (Group 8), showed no effect on retest score compared to Group 5 which received no drug in any test ( $F(7/432) < 1$ ). This showed anisomycin was not having a performance effect at retest. Comparisons of retest scores between snails that had received drug in both training and immediately after training (Groups 1 and 4) with snails that had received drug in training only showed no significant difference ( $F(7/432) < 1$ ). This was also found when Groups 1 and 4 were compared with snails that received the drug straight after training ( $F(7/432) < 1$ ). Thus there was no greater amnesia from multiple injections than evidenced by protein synthesis inhibition during training only ( $F(7/432) < 1$ ), or after training only ( $F(7/432) < 1$ ). Inhibition of protein synthesis after this period had no effect on long term habituation (see Group 5 compared with Group 8). Comparisons between groups that received anisomycin in retest (Groups 1, 2, 7, and 8) with those that did not (Groups 3, 4, 5, and 6) showed the anisomycin did not have a performance effect at retest ( $F(7/432) < 1$ ), and Comparison 14 showed that multiple injections did not have a cumulative effect on performance ( $F(7/432) < 1$ ). Comparison 12 (Groups 9 to 12, 14 and 15, with Group 13) further demonstrated that the amnesic effect of the antibiotic could not be attributable to long term latency action on performance.

Comparisons were also made between trained groups and

non trained groups that had received the same injection protocols to demonstrate that anisomycin was not having a long term latency effect. The eight training groups were each compared to the no training groups with the same treatment. There were no significant differences between retest scores in any comparison ( $F's(7/432) < 1$ ) except Group 5 with 14 ( $F(7/432) = 419.60, p < .001$ ), and Group 8 with 16 ( $F(7/432) = 459.87, p < .001$ ).

The amnesic effects at retest of injections during, or straight after training cannot be attributed to state dependency as disruption of long term habituation was also evident from the post training injection groups (Group 6 and 7) state dependency effects are further negated by the comparison of retest scores from Groups 1 and 2 with Groups 3 and 4 ( $F(7/432) < 1$ ), also Groups 5 and 6 with Groups 7 and 8 ( $F(7/432) < 1$ ).

## DISCUSSION

There was no effect on short term habituation from any dose of Anisomycin (2.5yg/snail, 25 yg/snail, or, 250 yg/snail) when injected 30 mins before training. Long term habituation was disrupted at all doses with either or both pre-training injections of drug or injection immediately post-training. Injection just prior to retest had no effect on retention. The results of Experiment 3 are directly comparable to those found with the other two antibiotics in Experiment 1 and 2.

## GENERAL DISCUSSION

The three antibiotic drugs actinomycin D, anisomycin, and puromycin all reversibly inhibit protein synthesis in the snail (see Chapter 7). The results reported in this chapter demonstrate that these drugs at a range of doses have no effect on short term habituation of the dorsal antennae withdrawal response of the snail Helix aspersa. However, at these doses these PSIs prevent long term habituation. Disruption of long term habituation was found with all drugs when the drug was injected immediately prior to training or straight after training.

These results support concepts of differential storage processes in short and long term habituation (e.g. Goelet et al., 1986; Gibbs & Ng, 1977). Such a concept has been reviewed in Chapter 2, and is further discussed in Chapter 18. The suggestion of differential processes, one protein synthesis independent (short term), and one protein synthesis dependent (long term), supports the eight behavioural experiments reported in Chapters 5 and 6.

Miller & Springer (1973) have argued that amnesic drugs disrupt storage of learning rather than retrieval. Results presented in this chapter suggest, for all three antibiotics, the PSI disruptable process is long term storage rather than retrieval. Inhibition of protein synthesis in retest had no effect on long term habituation provided protein synthesis had not been disrupted during or shortly after training. The issue of storage versus retrieval as the sensitive process for protein synthesis

inhibition has been confused by different use of terminology. Some authors see storage and retrieval as necessarily part of the same process (e.g. McGaugh and Gold), while others differentiate between them, but define neither adequately (e.g. Miller and Springer). While the results presented in this chapter support a hypothesis of storage disruption, the possibility of drugs having a long latency effect on retrieval remains. The comparison between drug, saline, saline group, with saline, saline, drug conditions does not necessarily rule out such effects, however, storage does appear to be the PSI sensitive process.

The 16 group design proved a useful contribution to this area of research. As well as establishing that the drugs had no effect on short term but could disrupt long term habituation, the design employed here negated three alternative explanations of such a long term amnesia; namely, state dependent effects, performance effects, and drug latency effects. This design could further serve other pharmacological or immunological manipulations of learning and memory to distinguish between the main effects of a manipulation and any possible alternative interpretation of an observed effect. The finding that the drugs did not disrupt long term habituation if injected immediately before retest, yet disruption was evidenced from injections immediately post training is of considerable consequence to this area of research. This result suggests a time window effect when protein synthesis is required. The time of effect of protein synthesis inhibition on this response is

further investigated in a later chapter (see Chapter 13).

With regard to dose effects, the above results support those reported in Chapter 9 in that there was again no effect of dose on degree of amnesia. All drugs at all doses produced the amnesic effect on long term habituation provided the drug was active in the suggested time window. Further to this point, the same main effects on short and long term habituation were found in both laboratory bred snails (Chapter 9) and in wild collected snails utilised in the three experiments reported in this chapter. This supports the robust nature of the main effect and also pertains to a recent alternative explanation of PSI induced long term amnesia; namely stress. Collected wild snails could be more stressed than laboratory bred snails. The effects of collection and housing stress and their possible interaction with long term amnesia are investigated in Chapter 12.

The results of the experiments reported in this chapter appear quite clear cut. However, trials to habituation is a measure of speed of habituation and may hide more subtle effects of drugs on development of habituation during a session. Consequently, a further experiment was conducted using measures of this development.

## CHAPTER 11

### THE EFFECTS OF PSIS ON THE DEVELOPMENT OF SHORT TERM HABITUATION AND ITS RETENTION WITH A FIXED TRIALS TRAINING PROCEDURE

The experiment reported in this chapter investigates the effects of the three antibiotics on short and long term habituation in order to provide a partial replication of experiments in Chapter 10 with a fixed number of trials per session design. The value and demerits of a fixed number of trials design have been discussed earlier (see Chapters 4 & 5). Here, it serves to explore the effects of the drugs on the development of habituation in training and in long term habituation. Since it is possible that the drugs have no effect on trial to habituation in short term habituation but show an effect in terms of individual responses, the following experiment investigates the effects of PSI on size and duration of initial response, and any drug effects on response decrements are explored graphically. Trials to criterion data were also recorded in this experiment to facilitate comparisons between this and the experiments of Chapter 10. As research in Chapter 9 and 10 show no effect of dose, only one dose of each of the three <sup>drugs</sup> anisomycin, actinomycin D and puromycin was used. The results for each measure are briefly discussed in terms of a replication of the main effects of Chapter 10 using different measures of habituation.

## METHOD

Subjects. Two hundred and forty mature snails of standard weight (8g) were sampled in the study. All subjects were from a population of 500 snails collected in wet condition from the Bovisand Bay area of Plymouth and laboratory housed as reported in Experiment 1, Chapter 3.

Procedure. Thirty snails were allocated to one of eight experimental conditions (see Table 11.1) which were the same as the first eight conditions of the experiments reported in Chapter 10. Of these 30 animals, if in the drug treatment, 10 received anisomycin (25yg/snail), 10 actinomycin D (25yg/snail) and 10 puromycin (25yg/snail). In saline treatment, all thirty received 0.1ml of physiological saline.

TABLE 11.1

### GROUP TREATMENTS

GROUP	TRAINING	REST 12 HOURS	RETEST
1	DRUG	DRUG	DRUG
2	DRUG	SALINE	DRUG
3	DRUG	SALINE	SALINE
4	DRUG	DRUG	SALINE
5	SALINE	SALINE	SALINE
6	SALINE	DRUG	SALINE
7	SALINE	DRUG	DRUG
8	SALINE	SALINE	DRUG

The same comparisons as conducted on Groups 1 to 8 of Chapter 10 could thus be employed. With regard to these comparisons, Groups 9 to 16 of Chapter 10 were not replicated in this study as their inclusion in the three experiments reported in the previous chapter served their function, of interest here was the effects of PSIs on

different measures of habituation.

Training injections were given 20 mins prior to training, rest period injections were given immediately after last response, and retest injections were given 20 mins before the snail was due for retest. Rest periods and interval between injection and training/retest were spent in the home vivarium.

The same blind procedure as reported in Chapter 10 was followed in this experiment. Habituation stimulus, interstimulus interval, and apparatus orientation period were also as reported in Chapter 10. In training, each snail received 10 stimulus presentations and the 10 responses elicited were recorded in terms of their duration and magnitude as reported in Chapter 3. Similarly, at retest 12 hrs post training, each snail received 10 stimulus presentations and responses were measured as in training.

Within the confines of the fixed number of trials design the number of trials required to reach criterion of habituation were also recorded. Criterion of habituation was two consecutive non responses. Animals still responding at stimulus 9 were accorded a score of 10.

Snails that failed to emerge from their shells in the apparatus orientation periods preceding training or retest, or which failed to emerge after injection were replaced by 'substitute' animals. Twelve substitute snails were run in each condition. Four such substitutions were required, 2 from group 8, 1 from group 3 (actinomycin D) and 1 from group 4 (saline).

The experiment was conducted between 1800 and 1200 on



the days commencing June 27, 28, 29, and 30, 1986.  
 Temperature in the laboratory varied from 19 to 23 C.

## RESULTS

### ANALYSIS OF INITIAL RESPONSE DURATION IN EACH TEST

TABLE 11.2

DURATION OF INITIAL TRAINING AND RETEST RESPONSES  
IN EACH TREATMENT CONDITION FOR THE THREE PSIS

GROUP	TREAT	TRAINING			RETEST		
		ANI	ACT	PURO	ANI	ACT	PURO
1	DDD	19.2	30.3	29.6	22.2	27.5	31.2
2	DSD	26.4	22.9	34.3	18.7	22.5	20.0
3	DSS	34.1	16.8	22.4	20.7	18.3	30.6
4	DDS	34.5	20.6	19.2	29.9	20.4	10.7
5	SSS	29.4	20.9	23.1	7.1	9.7	8.3
6	SDS	25.2	20.6	28.3	20.9	20.4	27.6
7	SDD	30.0	23.6	18.5	29.4	20.5	16.2
8	SSD	24.7	23.2	24.6	9.0	5.7	7.5

A 3-way ANOVA was conducted on the initial response duration in both the training and retest with a within subject factor of test (training/retest) and between subject factors of drug and condition (group 1 to 8). This revealed again, no significant effect of drug ( $F(2/216) < 1$ ) but a significant effect of condition ( $F(7/216) = 13.791, p < .001$ ) and test ( $F(1/216) = 235.799, p < .001$ ) and a significant condition x test interaction ( $F(7/216) = 72.512, p < .001$ ). All other interactions failed to reach significance ( $p > .5$ ).

Analysis of simple main effects revealed no significant difference in initial response duration in training between groups ( $F(7/216) < 1$ ) but a significant difference in first response at retest ( $F(7/216) = 123.673, p < .001$ ).

Multiple comparisons based on those conducted in Chapter 10 revealed protein synthesis inhibition had no

effect in short term habituation. Snails which received no drug in training (Groups 5 to 8) showed no difference in initial response duration to Groups 1 to 4) ( $F(7/216) < 1$ ). Thus, PSIs had no effect on initial response duration in short term habituation.

Within group comparisons revealed significant retention in Groups 5 and 8 ( $F(2/216) = 974.97$ , and  $965.89$ ,  $p < .001$ ), evidenced by a saving in initial response at retest. In all other groups which had received drug either in training, and or in retest, there was no retention ( $F(2/216) < 1$ ).

Initial response analysis also revealed retest scores in Groups 5 and 8, showed smaller first responses at retest than groups which received drugs in training (Groups 2 and 3) ( $F(7/216) = 803.56$ ,  $p < .001$ ), drug immediately after training (Groups 6 and 7) ( $F(7/216) = 768.83$ ,  $p < .001$ ), and snails that received drugs in both training and retest (Groups 1 and 4) ( $F(7/216) = 698.89$ ,  $p < .001$ ). Similarly, snails that received drugs in retest only (Group 8) showed no effect on initial retest response compared to Group 5 ( $F(7/216) < 1$ ).

Further multiple comparisons as outlined in Experiment 1, Chapter 10 again ruled out explanations of the effects of the PSIs as state dependency or performance effects utilising this measure. These comparisons are not fully reported as they show the same effects as reported in Chapter 10.

## ANALYSIS OF INITIAL RESPONSE MAGNITUDES IN EACH TEST

TABLE 11.3

MEAN CATEGORY OF FIRST RESPONSE MAGNITUDE IN SHORT  
AND LONG TERM HABITUATION IN EACH CONDITION

GROUP	TREAT	TRAINING			RETEST		
		ANI	ACT	PURO	ANI	ACT	PURO
1	DDD	2.5	2.6	2.5	2.4	2.6	2.4
2	DSD	2.9	2.3	2.6	2.6	2.1	2.4
3	DSS	2.5	2.3	2.4	2.3	2.4	2.3
4	DDS	2.4	2.4	2.5	2.6	2.2	2.3
5	SSS	2.3	2.3	2.7	1.9	1.6	1.7
6	SDS	2.2	2.9	2.5	2.5	2.5	2.5
7	SDD	2.7	2.4	2.5	2.6	2.4	2.7
8	SSD	2.4	2.5	2.4	1.8	2.0	1.9

Initial response magnitude from each trial, (training and retest) was analysed in a 3-way ANOVA which revealed a significant effect of condition ( $F(7/216) = 4.984, p < .05$ ) and test ( $F(1/216) = 25.000, p < .001$ ) with a significant condition  $\times$  test interaction ( $F(7/216) = 5.408, p < .05$ ). There was no effect of drug type ( $F(2/216) < 1$ ). All other interactions failed to reach significance ( $p > .5$ ).

Analysis of simple main effects revealed no difference between groups in training ( $F(7/216) < 1$ ), but a significant difference in retest ( $F(7/216) = 14.938, p < .001$ ). Multiple comparisons revealed within each group, there was only a saving in initial retest response magnitude in groups 5 and 8 ( $F(1/216) = 23.68$  and  $29.02, p < .001$  respectively) which had received no drug in either training or immediately after. All other groups showed no significant difference between training and retest ( $F(1/216) < 1$ ). Again, injection of the drug just prior to retest (group 8) had no effect on

initial response magnitude at retest ( $F(7/216) < 1$ ).

Results using this measure, again showed the same effects as found with response duration.

#### ANALYSIS OF TRIALS TO CRITERION IN EACH TRIAL

TABLE 11.4

#### MEAN TRIALS TO HABITUATION IN TRAINING AND RETEST IN EACH EXPERIMENTAL CONDITION FOR EACH PSI

GROUP	TREAT	TRAINING			RETEST		
		ANI	ACT	PURO	ANI	ACT	PURO
1	DDD	6.7	7.6	7.3	7.2	6.9	6.7
2	DSD	7.7	7.1	7.3	7.1	7.0	6.7
3	DSS	6.7	7.0	7.2	6.1	6.7	6.9
4	DDS	7.1	7.2	7.0	7.3	8.1	6.4
5	SSS	6.6	7.3	6.9	4.4	4.7	4.3
6	SDS	7.5	7.0	7.1	7.0	6.7	6.9
7	SDD	7.7	7.0	7.3	7.1	7.4	7.5
8	SSD	7.1	7.4	7.1	4.1	4.2	4.6

A 3-way ANOVA was also conducted on the trials required to habituate in training and in retest. This again revealed a significant effect of condition ( $F(7/216) = 34.333$ ,  $p < .001$ ) and trial ( $F(1/216) = 263.223$ ,  $p < .001$ ) with a significant condition x trial interaction ( $F(7/216) = 60.852$ ,  $p < .001$ ). There was no effect of drug type ( $F(2/216) < 1$ ). All other interactions failed to reach significance ( $p > .5$ ).

Analysis of simple main effects revealed no significant difference in trials to habituation between groups in training ( $F(7/216) < 1$ ), but a significant difference in retrials to habituation in retest ( $F(7/216) = 42.53$ ,  $p < .001$ ). Multiple comparisons revealed no difference in

trials to habituation in training and retest within Groups 1, 2, 3, 4, 6, and 7 ( $F(7/216) < 1$ ). There was a significant difference in Groups 5 and 8 which did not receive drug in either training or immediately after training ( $F(7/216) = 48.91$  and  $55.96$ ,  $p < .001$  respectively), these conditions showed retention

#### ANALYSIS OF THE DEVELOPMENT OF SHORT AND LONG TERM HABITUATION UNDER THE INFLUENCE OF THE THREE ANTIBIOTICS.

As statistical analysis of the decrement in responses over iterate stimulus presentations in training and retest was extremely unwieldy, they are reported graphically for drug effects on responses. These results are illustrated in Figure 11.1. & 11.2. There was no difference in terms of development of short term habituation, but a large variation between drug and saline treated snails in long term habituation.

Figure 11.1 compares groups 1 to 4 with groups 5 to 8, i.e. drug in training compared to saline in training. Figure 11.2 compares retest data (long term habituation) from Groups 2 and 3, drug in training but not immediately after training, Groups 6 and 7 drug immediately after training but not during, Groups 1 and 4, with drug in training and immediately after, and Group 5 and 8, with saline in both training and immediately after training.

FIGURE 11.1

DRUG EFFECTS ON RESPONSE DECREMENT IN TRAINING

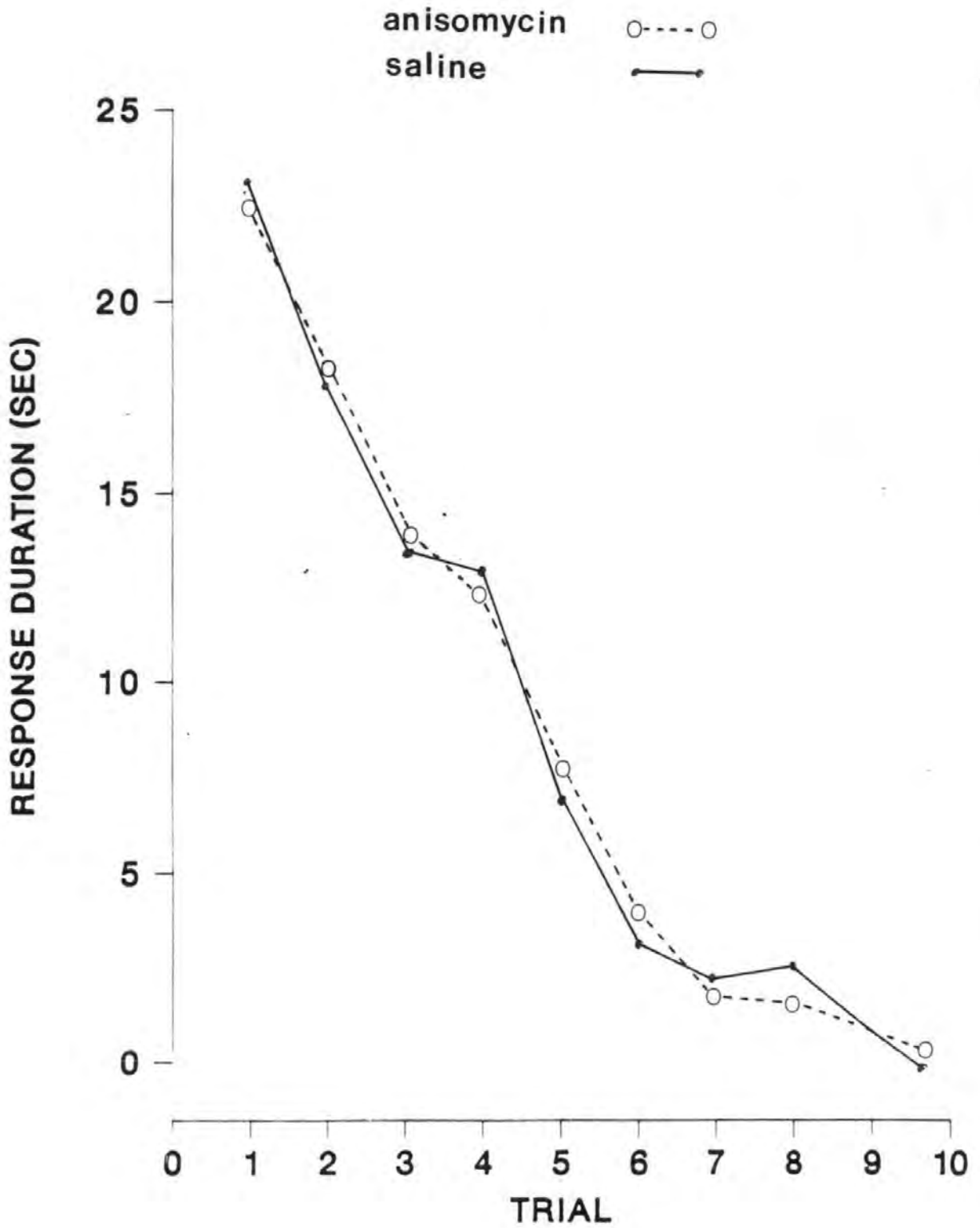
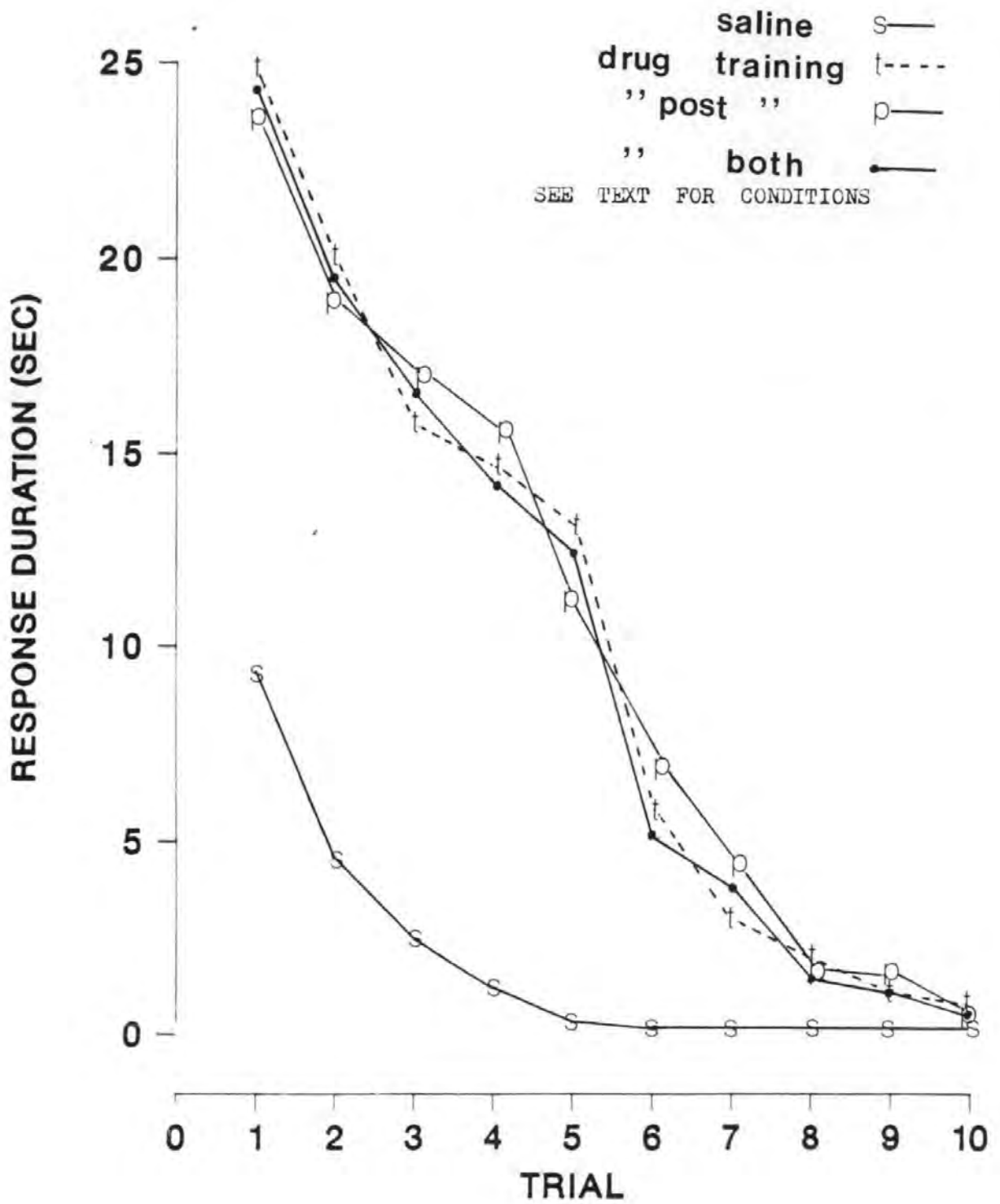


FIGURE 11.2

DRUG EFFECTS ON RESPONSE DECREMENT

AT RETEST



SEE TEXT FOR CONDITIONS

## GENERAL DISCUSSION

The above experiment again replicated the general findings of earlier experiments in this thesis. There was no effect of any of the three antibiotics on short term habituation. There was no difference between any of the three drug injected groups and saline injected groups in terms of response durations or magnitudes and each showed a normal decrement with iterate stimulus presentations. Similarly, there was no difference between groups in terms of trials to habituation.

Comparison of long term habituation in the 12 hr retest showed no evidence of long term habituation in groups that had received any of the three drugs in either training or immediately after training (Groups 1, 2, 3, 4, 6, and 7). There was no difference in level of amnesia between groups that received two drug injections within this time window and those that received one drug and a saline injection. These snails were found to show no difference in response duration, magnitude, or trials to habituation in retest compared with their initial training. Normal retention was evidenced in snails that received no drug in training or immediately after. These snails showed smaller responses throughout retest than they did during training. They also showed a saving in trials to habituation at retest compared to their original scores in training. Inhibition of protein synthesis by anisomycin, actinomycin D or puromycin just prior to retest had no effect on long term habituation (group 8). This provides further support for a critical time



period hypothesis where new protein synthesis is required for long term habituation (see Chapter 10).

Researchers have concluded from a variety of learning paradigms that protein synthesis inhibition has no effect on short term habituation (see Chapter 1 for review). However, these studies have relied on measures of learning restricted to time of acquisition of a task or number of trials required to achieve a criterion of learning. Unfortunately, no laboratories have reported effects of PSI's on the parametric characteristics of such short term learning. The experiment reported in this chapter goes some way to achieving this. There was not only no difference in short term habituation between drug and saline treated snails in terms of trials to habituation, but, also, drug treated snails showed a normal decrement of response durations and magnitudes.

Research reported thus far has used both laboratory bred snails and wild captured snails from a variety of sites to ensure replicability of the results. To ensure that the effects of PSI's are not the products of, or an interaction with, stress induced by laboratory conditions, a further investigation was carried out to investigate potential stress effects.

## CHAPTER 12

### STRESS: AN ALTERNATIVE EXPLANATION OF THE EFFECTS OF PSI'S ON SHORT AND LONG TERM HABITUATION

Before it can be unequivocally established that the amnesic effect is attributable to the inhibition of protein synthesis during a critical period, one further criticism of PSI research must be addressed. It has been suggested by some authors that the supposed amnesic effect of these antibiotics in rats and goldfish could be attributable to stress caused by handling, housing, learning paradigm, or laboratory conditions. Consequently, two further experiments were conducted. One compared the effects of protein synthesis inhibition on short and long term habituation on field tested animals in their natural habitat without the stress of collection or laboratory housing, on snails tested in the laboratory, and on snails tested in the field but still handled on test apparatus. The second investigated the effects of PSI treatment itself on habituation in the snail, investigating if the animal could show a change stimulus effect as established in Chapter 3 when PSI had been injected prior to training.

#### EXPERIMENT 1

Recent research in Germany (Laudien, Freyer, Erb, & Denzer, 1986) on goldfish in a shuttle-box experiment using active avoidance and a positive reinforcement test (food

reward in a colour discrimination task) reported an amnesic effect with cycloheximide only in fish exposed to isolation stress for 1 day. There was no amnesic effect in stress free cycloheximide treated fish. These authors suggest that the amnesic effect of the inhibitor was caused by stress induced by isolation of the fish. Unfortunately this remains the only empirical study of a laboratory stress effect on pharmacological disruption of long term learning/memory.

Unfortunately, the nature of PSI experimentation will necessarily stress subjects. Consequently, any potential major external stressor such as collection or housing of subjects should be investigated.

The ecological success of snails makes them readily available, and, consequently, like many invertebrate experimental animals, they are collected from the wild. This collection and subsequent housing in a glass vivaria may stress the snail. The findings of Laudien et al. (1986) necessitates a comparison of PSI effects in the snail between field tested and laboratory tested snails. Such a comparison is facilitated by the simple learning paradigm developed and its applicability to field research (Ray, 1986b).

From the research reported in Chapters 7, 8, 9, 10, and 11, it appears that all three antibiotics cause the amnesic effect. Therefore, only one antibiotic, anisomycin, was used. The employment of only one antibiotic was necessitated by the time consuming nature of field testing animals.

## METHOD

Subjects. Three hundred and twenty snails of standard Weight (8g) were employed. All subjects originated from the Devonport area of Plymouth.

Procedure. Twenty snails were allocated to each of the 16 experimental conditions described in Chapter 10 (see Table 10.1). The twenty snails were allocated to 4 equal groups (n=5). These were as follows:

- 1) Snails tested in the field on a laboratory board
- 2) Snails tested in the field in situ where located
- 3) Snails tested in laboratory on a laboratory board
- 4) Snails tested in laboratory in situ where located on the laboratory bench

Field tested snails were located, given the required injection according to group, numbered, and, 30 min later, placed on laboratory test board next to the site of location. After an apparatus orientation period of 1 min, training to habituation commenced. No-training groups were still handled, injected, and then placed on test board but received no stimulus exposure. Retest procedure was as for training. The interval to retest was spent in the animals natural habitat. Retest interval for all groups was 12 hours.

Field unmolested snails were located, injected, numbered, and replaced where they were found. Training to habituation was conducted by each stimulus being applied

wherever the animal was situated when the stimulus was due. No-training groups were injected then returned to where they were found.

Laboratory tested snails were housed as reported in Experiment 1, Chapter 3. Each snail was removed from home vivarium, injected, and then returned for the 30 min, pre-training interval. Training to retest interval was also spent in the home vivarium. Training and retest was conducted on a test board (after an apparatus orientation period. No-training groups were injected and placed on test apparatus at appropriate times but received no training. The interval between training and retest was spent in the home vivarium.

Laboratory unmolested snails were located from home vivaria, injected and placed on a laboratory bench 3 x 2 m surrounded by 10 cm vertical boarder. All stimuli were presented where the snail was found on the bench, so the only handling was in injection. No-training groups remained in situ without stimuli. Training to retest interval was spent in situ on the bench.

With all four groups, in each of the 16 experimental conditions, ordering of experimentation between groups was alternated on each night. The experiment occurred overnight between 2000 and 1245 Hrs on the mornings of July 5, 6, 7, 8, 9, 11, 12, 15, 17, 18, 19, and 20, 1986. Air temperature varied from 10 to 13 C and laboratory temperature varied from 17 to 20 C.

Habituation stimulus, equipment and ISI for all groups in all conditions was as reported in Experiment 1, Chapter

10. All animals used in this study were collected at the end of the study and housed in a Plymouth garden for 1 month to study drug treated snails for mortality.

## RESULTS

TABLE 12.1

MEAN TRIALS TO HABITUATION IN EACH GROUP IN ALL FOUR  
EXPERIMENTAL CONDITIONS

CONDITION	TRAINING				RETEST			
	FIELD	FIELDU	LAB	LABU	FIELD	FIELDU	LAB	LABU
1	9.8	12.0	11.6	8.6	9.2	10.2	10.8	13.0
2	9.0	9.6	11.4	13.0	9.2	10.2	10.9	12.6
3	10.4	12.2	11.4	11.4	11.4	12.4	11.4	9.8
4	10.4	11.0	11.2	10.4	9.6	10.6	11.6	9.9
5	10.8	9.8	11.4	11.6	6.2	6.2	7.3	6.3
6	8.8	9.8	9.1	12.4	10.2	10.8	10.4	10.8
7	10.4	12.0	9.8	11.4	10.4	12.6	11.0	12.6
8	11.4	9.2	10.0	9.8	6.0	5.2	7.0	5.2
9	-	-	-	-	10.2	10.0	13.0	10.0
10	-	-	-	-	10.0	10.4	11.8	10.8
11	-	-	-	-	10.0	9.0	12.2	10.0
12	-	-	-	-	11.4	11.0	10.4	10.0
13	-	-	-	-	10.2	10.2	10.4	10.2
14	-	-	-	-	9.2	10.4	10.2	10.0
15	-	-	-	-	10.2	9.2	10.2	10.6
16	-	-	-	-	9.4	10.6	11.4	10.4

\*FIELD = FIELD MANIPULATED      LAB = LABORATORY MANIPULATED  
FIELDU = FIELD UNMOLESTED      LABU = LABORATORY UNMOLESTED

The results are illustrated in Table 12.1. Analysis of trials to habituation in retest in a 2-way between subjects ANOVA revealed a significant effect of group ( $F(15/256) = 8.624, p < .001$ ). There was no difference between any of the four experimental settings ( $F(3/256) < 1$ ) and no interaction ( $F(45/256) < 1$ ).

A further 3-way ANOVA, with a within subject factor of

test and between subject factors of group and experimental setting, was conducted on snails that received training and a subsequent 12 hr retest (Groups 1...8) to compare the training and retest scores. This showed a significant effect of Group ( $F(7/128) = 5.326, p < .001$ ), Test ( $F(1/128) = 29.408, p < .001$ ) with a significant Group x Test interaction ( $F(7/128) = 24.947, p < .001$ ). There was no difference between snails in different experimental settings ( $F(3/128) = 1.713, p < .05$ ). All other interactions failed to reach significance.

The above results show that the environment where a snail was tested had no effect on the main effects of PSIs on short and long term habituation. However, further comparisons were conducted to ensure that there were no state dependency, performance or drug latency effects in these results (see Chapter 9).

Further analysis of simple main effects and by multiple comparisons revealed that with regard to short term habituation, groups 1 to 4 showed no effect of drug in training compared with saline injected snails (groups 5 to 8) ( $F(7/128) < 1$ ). It was concluded from this that anisomycin had no effect on short term habituation in any environment. The same comparison ruled out the interaction of stress with short term drug latency effects.

Within group comparisons revealed significant retention in groups which received no drug in either training or immediately after training, group 5 ( $F(7/128) = 23.71, p < .001$ ), and group 8 ( $F(7/128) = 19.84, p < .001$ ).

Comparisons between retest scores of group 5 and 8 with groups 13 and 16 showed retention of training over 12 hours

( $F(7/128) = 101.72, p < .001$ ).

Further analysis of retest scores revealed that retest scores in groups 5 and 8 which received no drug in training or immediately after training required fewer trials to habituation than snails that had received drug in training (groups 2 and 3) ( $F(7/128) = 46.92, p < .001$ ), drug immediately after training (group 6 and 7) ( $F(7/128) = 27.12, p < .001$ ), and snails that had received drug in training and immediately after training (group 1 and 4) ( $F(7/128) = 19.79, p < .001$ ). Similarly, snails that received drug in retest only (group 8) showed no effect on retest score compared to group 5 which received no drug in any test ( $F(7/128) < 1$ ). This showed the drug had no performance effect at retest, this was true in all environments. Comparisons of retest scores between snails that had received anisomycin in both training and immediately after training (group 1 and 4) with snails that received drug in training only showed no significant difference ( $F(7/128) < 1$ ). Thus there was no greater amnesia from multiple injections than evidenced by inhibition drug training only or after training only. Inhibition of protein synthesis after this period had no effect on long term habituation (see group 5 compared with group 8). Comparisons between snails that received drug in retest (groups 1, 2, 7, and 8) with those that did not (groups 3, 4, 5, and 6) showed the drug did not have a performance effect at retest ( $F(7.128) < 1$ ).

Comparisons were again made between trained groups and non trained groups that had received the same injection



protocols (see Chapter 9). There were no significant differences between retest scores in any comparison ( $F(7,128) < 1$ ) except groups 5 with group 14 ( $F(7/128) = 27.00, p < .001$ ) and group 8 with 16 ( $F(7/128) = 29.63, p < .001$ ).

The amnesic effects shown by all groups cannot be attributable to state dependency, as disruption of long term habituation was evident from post training injection groups (6 and 7). State dependency effects are further negated by the comparison of retest scores from Groups 1 and 2 with groups 3 and 4 ( $F(7/128) < 1$ ), also Groups 5 and 6 with groups 7 and 8 ( $F(7/128) < 1$ ).

#### LONG TERM MORTALITY STUDY RESULTS

Participating snails were also used in a long term mortality study. They were housed in a Plymouth garden for 1 month post experiment, and the number of dead and missing animals were calculated. It was found that 5 snails were dead after 1 month, 2 from the Drug, Saline, Drug group, 1 from Saline, Saline, Drug group, 1 from the Saline, Saline, Saline group and 1 from Drug, Drug, Drug group. Consequently, the protein synthesis inhibition with anisomycin was reversible and survivable with no very long term ill effects. Two snails from the Drug, Saline, Saline group were found to be missing, presumed lost.

## DISCUSSION

The results again showed anisomycin prevented long term habituation when active during or shortly after training. It had no effect on short term habituation in the same response. The amnesic effect occurred in all experimental settings including the minimal interference laboratory and field groups. The unmolested field test snails, though still subject to stress from injection and procedure, had not had the trauma of capture or housing stress. This result is interesting in light of recent Laudien et al. (1986) paper. The amnesic effect was not limited to high stress conditions e.g. laboratory test groups. It appears to occur irrespective of levels of stressing of the animal, and strongly argues against stress factors as an explanation of the effect.

## EXPERIMENT 2

### PROTEIN SYNTHESIS INHIBITION EFFECTS ON FINAL STAGES OF SHORT TERM HABITUATION

The initial experiment reported in this chapter investigated laboratory housing and collection stress in PSI experiments with snails. As stated in the introduction, PSI experiments by their nature will stress an animal to some extent. In order to investigate if the PSI induced stress interferes with the animal's ability to learn, a further experiment was conducted using one of the parametric

characteristics of learning established in Chapter 3; namely, the change stimulus effect. In order to support the initial experiment of this chapter, the effects of a change stimulus was sampled after training to criterion of habituation. This investigated whether a change stimulus would re-evoke and dishabituate the response, as demonstrated in Chapter 3, in the absence of protein synthesis. If normal change stimulus effects are demonstrated with PSI's, it would further decrease the viability of stress as an explanation of PSI effects.

#### METHOD

Subjects. Eighty mature Helix aspersa of standard weight (8g) were collected from Stonehouse area of Plymouth. Laboratory housing was as reported in Experiment 1, Chapter 3.

Procedure. Snails were allocated to one of two experimental conditions: a change condition and a no change condition.

Snails in the change condition were habituated to criterion with the aluminium prod, tactile stimulus (see Experiment 1, Chapter 3). Stimuli were presented with an ISI of 30 seconds. Thirty seconds after the third consecutive non response the snail received a water drop stimulus change stimulus (see Experiment 1, Chapter 3) followed, 30 sec later, by a return presentation of the training stimulus (aluminium prod).

Snails in the no change condition were habituated to

criterion with the aluminium prod with ISI 30 seconds, however instead of a change stimulus, 30 seconds after the last non response the snails received the tactile stimulus again and after a further 30 sec another presentation of the same stimulus. Comparisons between conditions were then made for the test stimulus (change/ no change) and the subsequent dishabituation stimulus. Responses were measured in terms of their duration and magnitude (see Chapter 3). Snails which failed to emerge within 60 sec of stimulus presentation were removed from the sample and replaced by substitutes. Only one such substitution was necessary. A double blind procedure the same as that in Experiment 1, Chapter 3 was used.

Within each of the above two experimental conditions, 10 snails received an injection of physiological saline, 10 actinomycin D, 10 anisomycin and 10 puromycin at the same dose as Experiment 1 of this chapter. The same blind procedure was applied. Injections were administered 30 minutes prior to training.

## RESULTS

Response duration data were analysed in a 3 way ANOVA with a within subject factor of trial and between factors of condition (change/no change) and pharmacological treatment. The trial factor consisted of initial response in training, Test stimulus 1 (change stimulus), and Test stimulus 2 (dishabituation). This revealed a significant effect of condition ( $F(1/72) = 93.214, p < .001$ ) and trial ( $F(1/36) =$

341.490,  $p < .001$ ) and a significant Condition  $\times$  trial interaction ( $F(1/72) = 325.595$ ,  $p < .001$ ). There was no effect of pharmacological treatment ( $F(3/72) < 1$ ). All other interactions failed to reach significance ( $p > .5$ ). The results are illustrated in Figures 12.2, 12.3, 12.4, and 12.5. Each figure demonstrates the effects of a particular pharmacological treatment.

Analysis of simple main effects showed there was no difference between response duration of first training response, test response, and dishabituation response in the change condition ( $F(1/72) < 1$ ). There was a significant difference across the three responses in the no change condition ( $F(1/72) = 166.663$ ,  $p < .001$ ) with a large initial training response but much smaller responses at the test and retest stimuli presentations. There was no difference between the two conditions at initial response ( $F(1/72) < 1$ ), but a significant difference at the change/no change test ( $F(1/72) = 72.766$ ,  $p < .001$ ), and at the dishabituation test response ( $F(1/72) = 74.339$ ,  $p < .001$ ). The change stimulus reinstated a large response that was absent in the no change test. Similarly, in the change condition, dishabituation was seen to occur.

Data were also analysed in terms of response magnitude in a 3-way ANOVA, and this showed a significant difference between conditions ( $F(1/72) = 289.773$ ,  $p < .001$ ) and trial ( $F(1/72) = 504.692$ ,  $p < .001$ ) with a significant Condition  $\times$  trial interaction ( $F(1/72) = 460.828$ ,  $p < .001$ ). All other interactions failed to reach significance ( $p > .5$ ). There was no significant difference between pharmacological treatments

FIGURE 12.2

# ACTINOMYCIN D

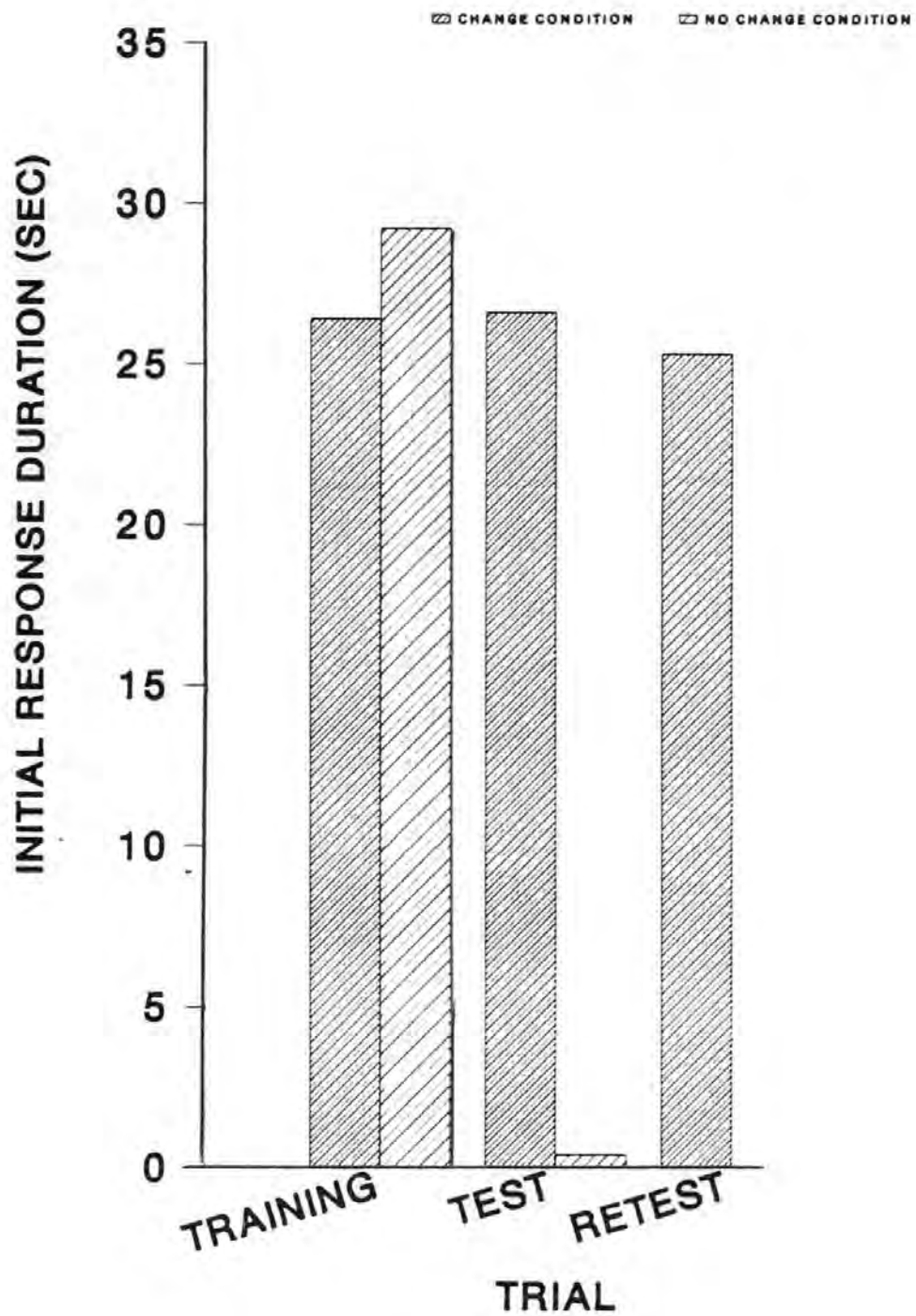


FIGURE 12.3

# SALINE

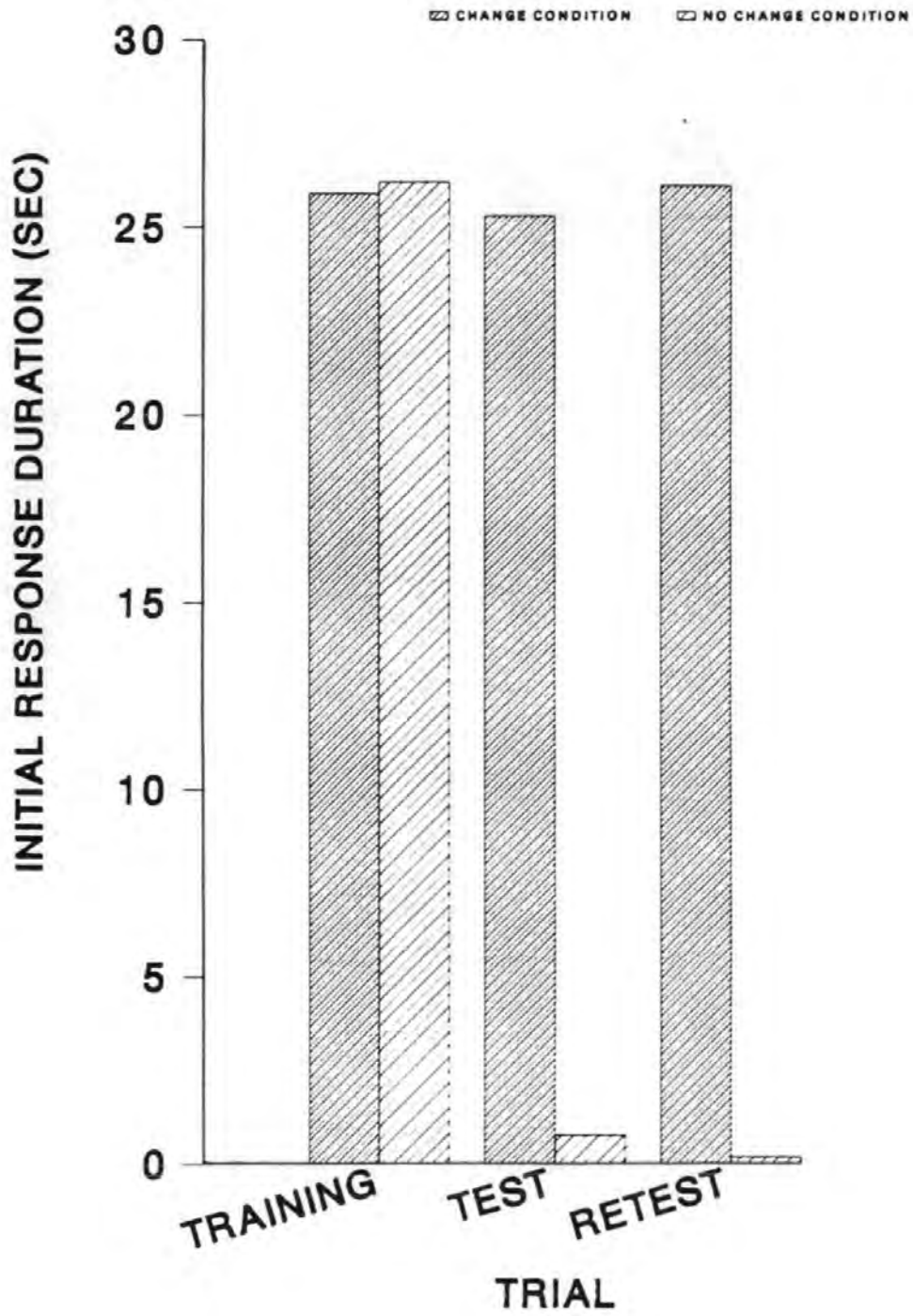


FIGURE 12.4

# ANISOMYCIN

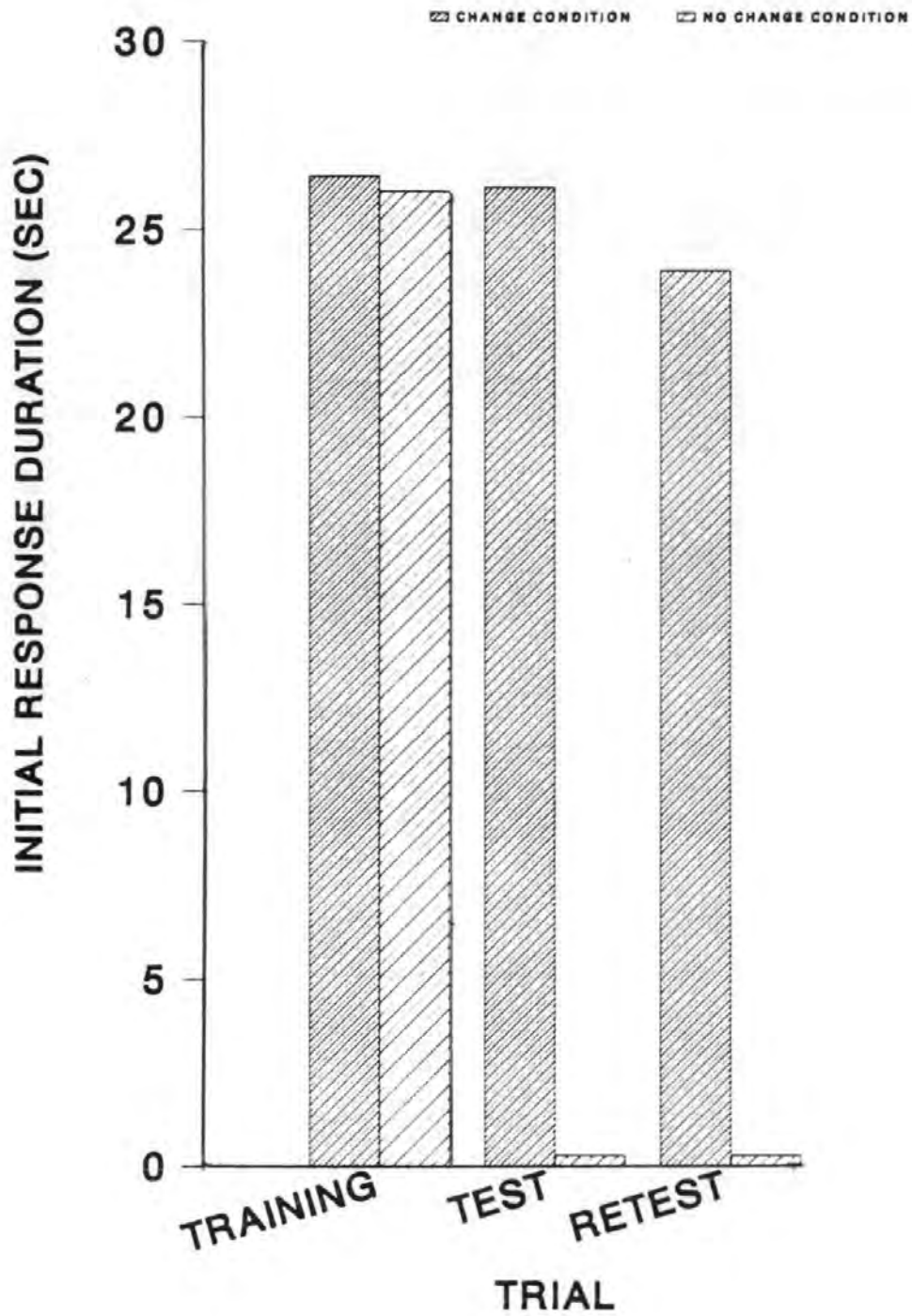
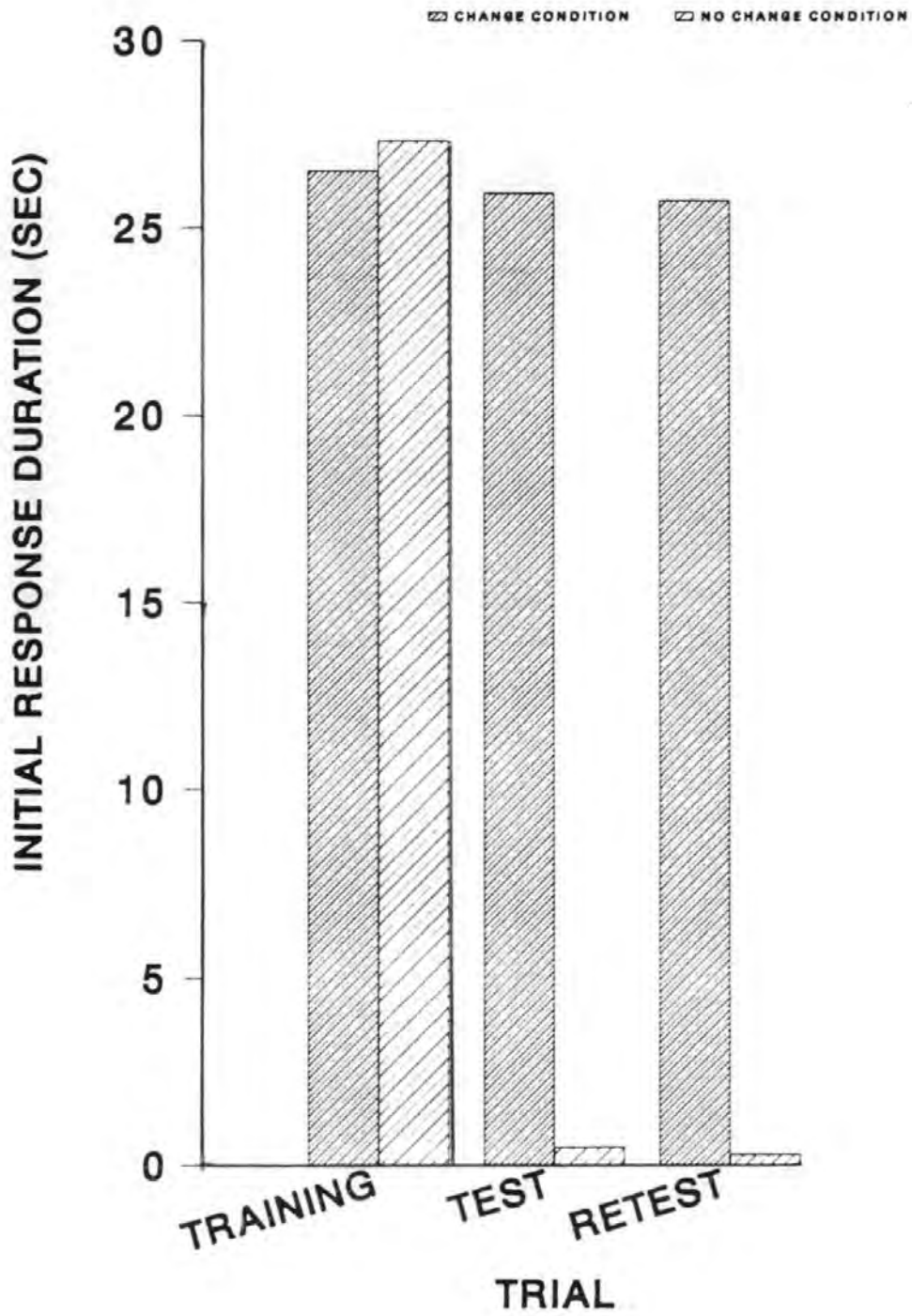




FIGURE 12.5

# PUROMYCIN



( $F(3/72) < 1$ ).

Analysis of simple main effects for this measure revealed that, within the change condition, there was no significant difference between initial training response, change/no change test, and dishabituation test ( $F(1/36) < 1$ ) in the change condition, but, a significant difference in the no change ( $F(1/36) = 241.249, p < .001$ ) with much smaller responses in both tests compared to initial training magnitude ( $F's(1/36) = 310.08, \text{ and } 361.75, p < .001$  respectively). Further, there was no difference between the two conditions at initial training response ( $F(1/36) < 1$ ), but a significant difference at the change/no change test ( $F(1/36) = 116.997, p < .001$ ) and a significant difference in the dishabituation test ( $F(1/36) = 120.137, p < .001$ ). Again, on this measure, the change stimulus reinstated a large response magnitude. Such an effect was absent in the no change group at test, and, in the change group, dishabituation was seen to occur.

## DISCUSSION

None of the three antibiotics had any effect on short term habituation. In all drug conditions snails showed a reinstatement of responding to a change stimulus after criterion of habituation had been achieved. This suggests that short term habituation showed normal parametric characteristics in the absence of protein synthesis and the learning was not affected by the stress caused by the learning paradigm or drug treatment. The effects of

antibiotics on other parametric characteristics of habituation will be discussed in later chapters (see Chapters 14, and 15).

### GENERAL DISCUSSION

The above two experiments investigated the stress of the experimental paradigm employed in PSI investigations of snail learning and addressed the question of differential levels of housing and collection stress on the long term amnesia observed in earlier chapters. It was found in Experiment 1 that long term amnesia was found in snails receiving drug during or immediately after training, and that this amnesia did not vary as a function of the degree of stressing the snails experienced.

Experiment 2 showed that any stress effects there might have been from PSI procedure were not sufficient to affect the change stimulus and dishabituation phenomena and provide further evidence of the lack of PSI effects on short term habituation.

Results suggest a multiple process view of habituation where short term habituation is protein synthesis independent and long term habituation requires protein synthesis to be intact for a critical period in and shortly after training. Kandel has suggested that one problem of PSI research is the lack of replicability of results. Experiment 1 is the sixth replication of the main PSI effects thus far discussed.

It is interesting that the results suggest a critical

period for protein synthesis for long term habituation. Protein synthesis inhibition after this period has no effect on long term habituation. This critical period is investigated in the next chapter.

## CHAPTER 13

### A CRITICAL TIME WINDOW OF AMNESTIC EFFECTS OF ANISOMYCIN, ACTINOMYCIN D, AND PUROMYCIN AND THE EFFECTS OF DOSE ON SUCH TIME/EFFECT RELATIONSHIPS

Research reported thus far in this thesis has established anisomycin, actinomycin D and puromycin all reversibly inhibit protein synthesis in Helix aspersa (see Chapter 7). It has also established doses of each drug which have no debilitating effects on general snail behaviour (see Chapter 8), and that these same safe doses produce no effect on short term habituation when injected 30 minutes prior to training. However, anisomycin, actinomycin D, and puromycin do disrupt long term habituation when injected 30 minutes prior to training or immediately after training. They had no effect when injected just prior to the long term retest. This has been established with a variety of measures of habituation. These results suggest that amnesic effects are produced only with certain times of injection relative to training (see Chapters 9, 10, and 11). This time window effect was further investigated in this chapter.

Several studies have compared the extent and duration of cerebral protein synthesis inhibition with the degree of retrograde amnesia using antibiotic drugs. One such study (Flood et al., 1973) compared single injections of anisomycin and cycloheximide at doses that achieved similar levels of cerebral protein synthesis inhibition and reported greater amnesia in cycloheximide treated animals. However,

protein synthesis inhibition with cycloheximide was of greater duration than that with anisomycin. These authors further report an intensification of amnesia with successive injections of anisomycin at 2 hrs intervals up to 8 hrs post training. In a subsequent study, Flood et al. (1975) used multiple injections of anisomycin and reported an inhibition of protein synthesis in excess of 80% was necessary for 6 to 8 hours for amnesia to occur in mice. Squire & Davis (1975) used two different doses of Anisomycin (30 and 210mg/kg). Inhibition of cerebral protein synthesis of greater than 90% was necessary in the first 90 min following training to cause amnesia. The higher dose of anisomycin which was reported to slightly increase the inhibition of protein synthesis also increased the amnesia. More recent studies have claimed that a few minutes of incomplete inhibition of cerebral protein synthesis with anisomycin can suffice to prevent amnesia by subsequent treatment with anisomycin (Bennett et al 1977).

A more detailed study is provided by Quinton & Kramarcy (1977). They investigated the effects on passive avoidance behaviour of cerebral protein synthesis inhibition in mice of six different doses of cycloheximide which were administered at five different times. The lowest dose of cycloheximide observed to cause amnesia was 7 mg/kg, and this had to be administered 30 min before training. On the other hand 150 mg/kg, was amnesic when given up to 90 min before training. Inhibition of cerebral protein synthesis of greater than 88% at the time of training was necessary for amnesia. However, there was evidence that "memory mechanisms

may recover more rapidly following cycloheximide than does general protein synthesis". Several hours after administration of high doses of cycloheximide, protein synthesis inhibition was still in excess of 90%, and yet retention in mice trained at this time was higher than with lower doses of cycloheximide administered at shorter time points relative to training. Similar results were forthcoming in a subsequent study by Bloom et al. (1977).

Time of effect analysis of antibiotic induced amnesia suffers in the main from different laboratories using different antibiotics with different modes of action at different doses and at different times relative to training and retest. The limited work available does suggest that there is a critical time window during which long term memory requires the synthesis of new proteins, and this effective time window varies as a function of dose and antibiotic.

Further, some researchers suggest that for a brief period after training protein synthesis is necessary for long term learning to occur. PSI's can disrupt long term learning if injected up to an hour post training (e.g. Aggranoff, 1967, 1970; Goelet et al., 1986; Montarolo et al., 1986; Squire and Davis, 1984). However, the actual post-training limit of the critical effective period remains unclear and has not been investigated as a function of dose.

The critical period hypothesis for protein synthesis is of great importance to the interpretation of PSI results in terms of underlying mechanisms. Montarolo et al. (1986) have suggested that a single learning event initiates several

memory processes with different time courses of retention. While short term memory is said to involve covalent modifications of pre-existing proteins, long term memory requires the expression, during learning, of additional genes. In order to investigate such a hypothesis, a more unequivocal investigation of dose and time of effect relationship must be conducted.

There has also been a suggestion that since high inhibition of cerebral protein synthesis is necessary for amnesia, inhibition of cerebral protein synthesis simply depletes a protein important for normal cerebral function or depletes already existing proteins rather than having an effect at the genome (Crick, 1984). Bennett et al. (1977) have argued that neither acquisition nor retrieval are significantly impaired by amnesic doses of the inhibitors, and, for amnesia to occur, the inhibition of protein synthesis must be specifically related in time to the training.

Squire & Barondes (1976) considered the particular case of the depletion of a constitutive protein with a short half-life by antibiotics. Their data showed that cycloheximide did not cause significantly greater amnesia when given 5 min rather than 1 min before training, and more importantly, that inhibition of protein synthesis by more than 80% for 118 min before training was not amnesic. This important work has often been criticised in that it only used one antibiotic and it could be argued that constitutive protein could be resistant to cycloheximide.

The criticism of depletion of proteins necessary for



normal cerebral functioning has not been addressed with respect to the effects of PSIs on long term learning. However, data from Helix in the test battery (see Chapter 8) decrease the power of this criticism in that if normal cerebral functioning was disrupted, then normal behavioural repertoire of the animal would also be affected. Furthermore, it was demonstrated in Chapters 10, and 12 that there are no long latency effects of PSIs.

In an attempt to further explore this important and contentious aspect of PSI studies, a series of experiments are reported in this Chapter using three antibiotics.

#### GENERAL METHOD

Separate experiments were conducted on each of the antibiotics anisomycin, actinomycin D, and puromycin, to compare 3 doses of drug at 10 pre-training intervals by observing amnesic effects on short and long term habituation. The effects of 10 post-training intervals on long term habituation were also investigated. All three experiments used the following methodology.

Subjects. One thousand eight hundred snails of standard weight (8g) were collected from the Stonehouse area of Plymouth in wet conditions were used in each experiment; 600 in each dose analysis. Laboratory housing as reported in Experiment 1, Chapter 3.

Procedure. Ninety of the above snails were allocated to each of 20 injection time groups, 10 pre-training times and 10 post-training times as follows:

EXPERIMENTAL CONDITIONS

GROUP	PRE-TRAINING INTERVAL	GROUP	POST-TRAINING INTERVAL
1	120 MIN	11	1 MIN
2	80 MIN	12	10 MIN
3	70 MIN	13	20 MIN
4	60 MIN	14	30 MIN
5	50 MIN	15	40 MIN
6	40 MIN	16	50 MIN
7	30 MIN	17	60 MIN
8	20 MIN	18	70 MIN
9	10 MIN	19	80 MIN
10	1 MIN	20	120 MIN

Within each injection time group, the 90 snails were, subdivided into 3 smaller groups each involving one of 3 doses of drug. For each dose (2.5 yg/snail, 25 yg/snail, and 250 yg/snail), thirty snails were studied, 10 received drug at that dose, 10 received saline, and 10 received no injection. The experiments were analysed in terms of time of effect of drugs at each dose.

Each snail was removed from the home vivarium and injected, then replaced for the injection-training interval. Training procedure, habituation stimulus, criterion of

habituation, and ISI were as reported in Experiment 1, Chapter 9. Trials required to reach criterion of habituation were recorded for training and a retest which took place 8 hrs post training. Savings in retrials to habituation compared to trials to criterion in training were used to assess retention. The interval between training and retest was spent in the home vivaria.

This general methodology was employed in three experiments to look at the three antibiotics. Each experiment was analysed at each dose for the effects of pre-training injection time on short and long term habituation. Similarly within each dose, post training injection time was analysed for any effect on long term habituation.

## EXPERIMENT 1

### TIME OF EFFECT ANALYSIS FOR PUROMYCIN AT THREE DOSES

Results for groups in puromycin experiment are reported in Figures 13.1 to 13.9 and show a clear effect of time of injection on disruption of long term habituation. The Experiment was conducted on the nights of March 1 to 15, and 17 to 31, 1986 between 1600 and 0930; temperature in the laboratory varied from 16 to 19 C. Results from the 10 pre-training injection times are analysed first, followed by the snails in the 10 post-training conditions. This was conducted for each of three doses.

DOSE 1 (2.5yg/snail)

Analysis of the ten pre-training injection times in a 3 way ANOVA for the three hundred snails with pre-training injection times. The analysis had between subject factors of time (injection time), and condition (drug, saline or no injection) and a within subject factor of test (training/retest). This revealed a significant effect of time ( $F(9/270) = 17.04, p < .001$ ), condition ( $F(2/270) = 141.123, p < .001$ ) and test ( $F(1/270) = 1287.874, p < .001$ ). There were significant interactions of time x condition ( $F(9/270) = 15.275, p < .001$ ), time x test ( $F(9/540) = 14.080, p < .001$ ), condition x test ( $F(2/90) = 127.691, p < .001$ ), and time x condition x test ( $F(9/270) = 17.270, p < .001$ ).

Analysis of simple main effects by comparisons across conditions showed that puromycin had no effect on short term habituation ( $F(9/270) < 1$ ) (see Figure 13.1). Long term habituation was, however, absent in snails that received the drug at 40, 30, 20, 10 and 1 minute pre-training, there was no difference between trials to habituation at training and retest ( $F's(9/270) < 1$ ) (see Figure 13.2). Earlier injection times (120, 80, 70, 60, and 50 minutes pre-training) had no effect on long term habituation. Snails in these groups showed a saving in trials to habituation compared to retest ( $F's(9/270) = 785.02, 834.92, 799.52, 800.70, \text{ and } 796.78, p < .001$  respectively).

For the post-training injection groups a 3-way ANOVA showed significant effects of time ( $F(9/270) = 19.112, p < .001$ ), condition ( $F(2/270) = 138.731, p < .001$ ), and test

FIGURE 13.1

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 1)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

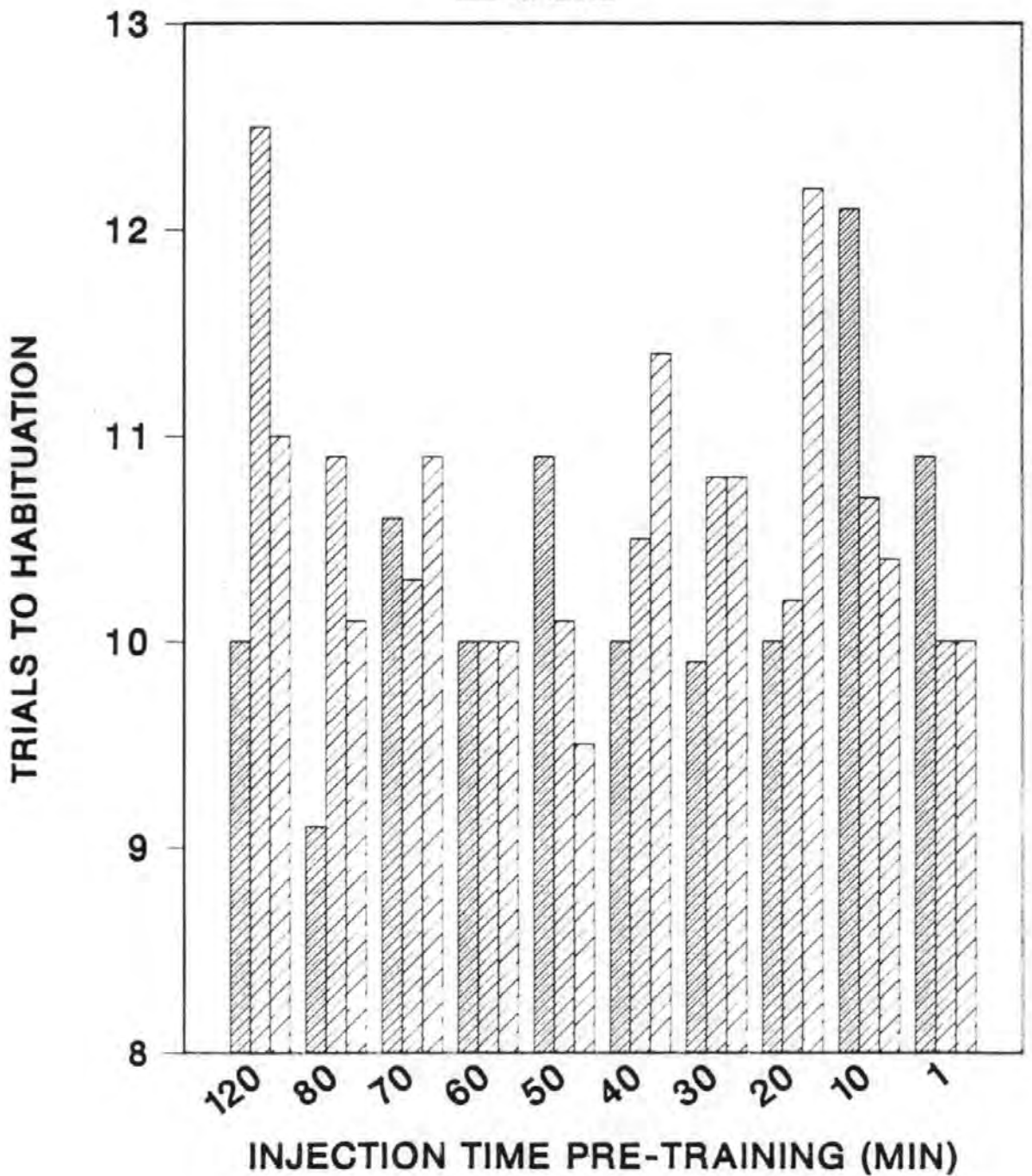


FIGURE 13.2

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

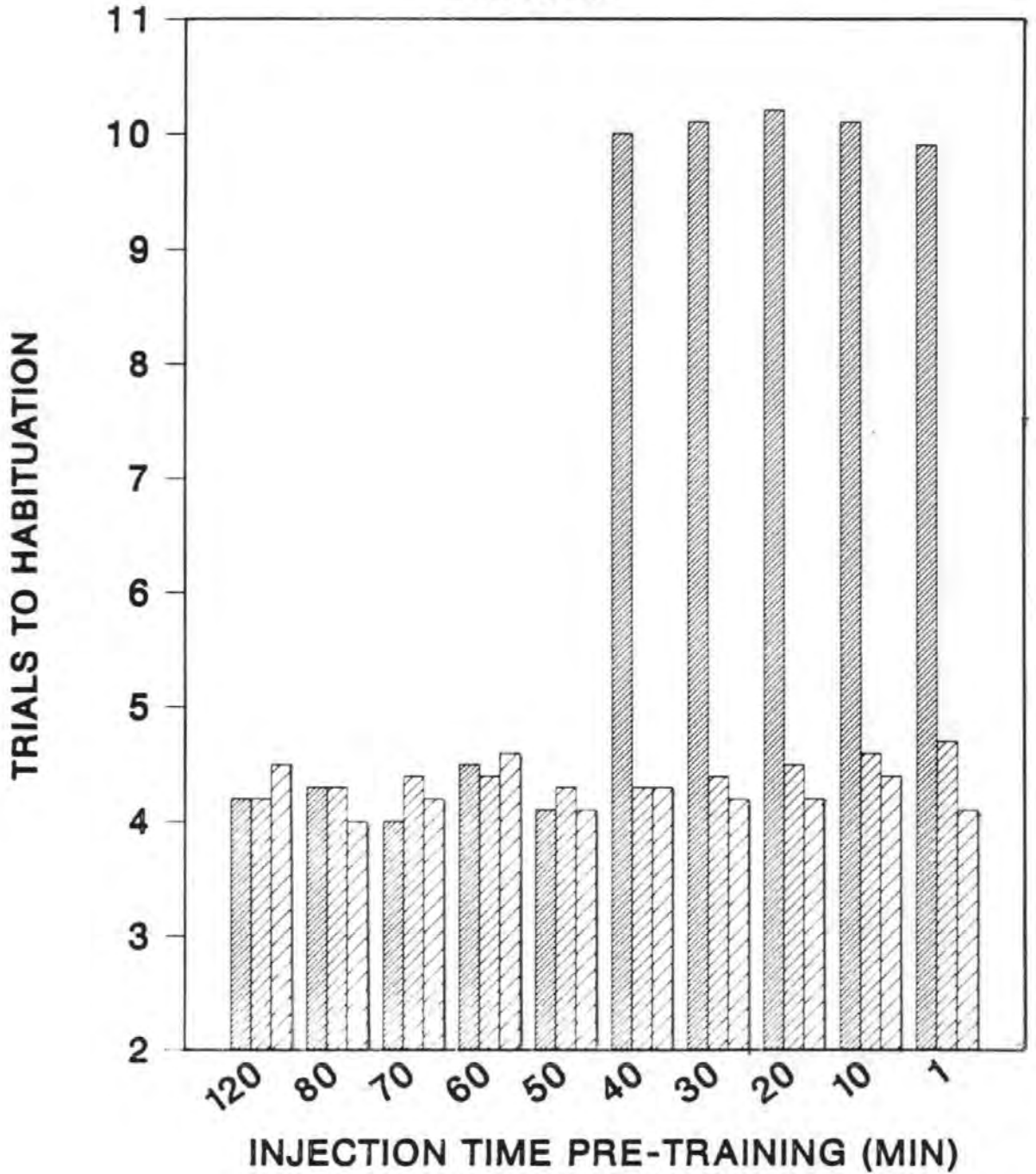
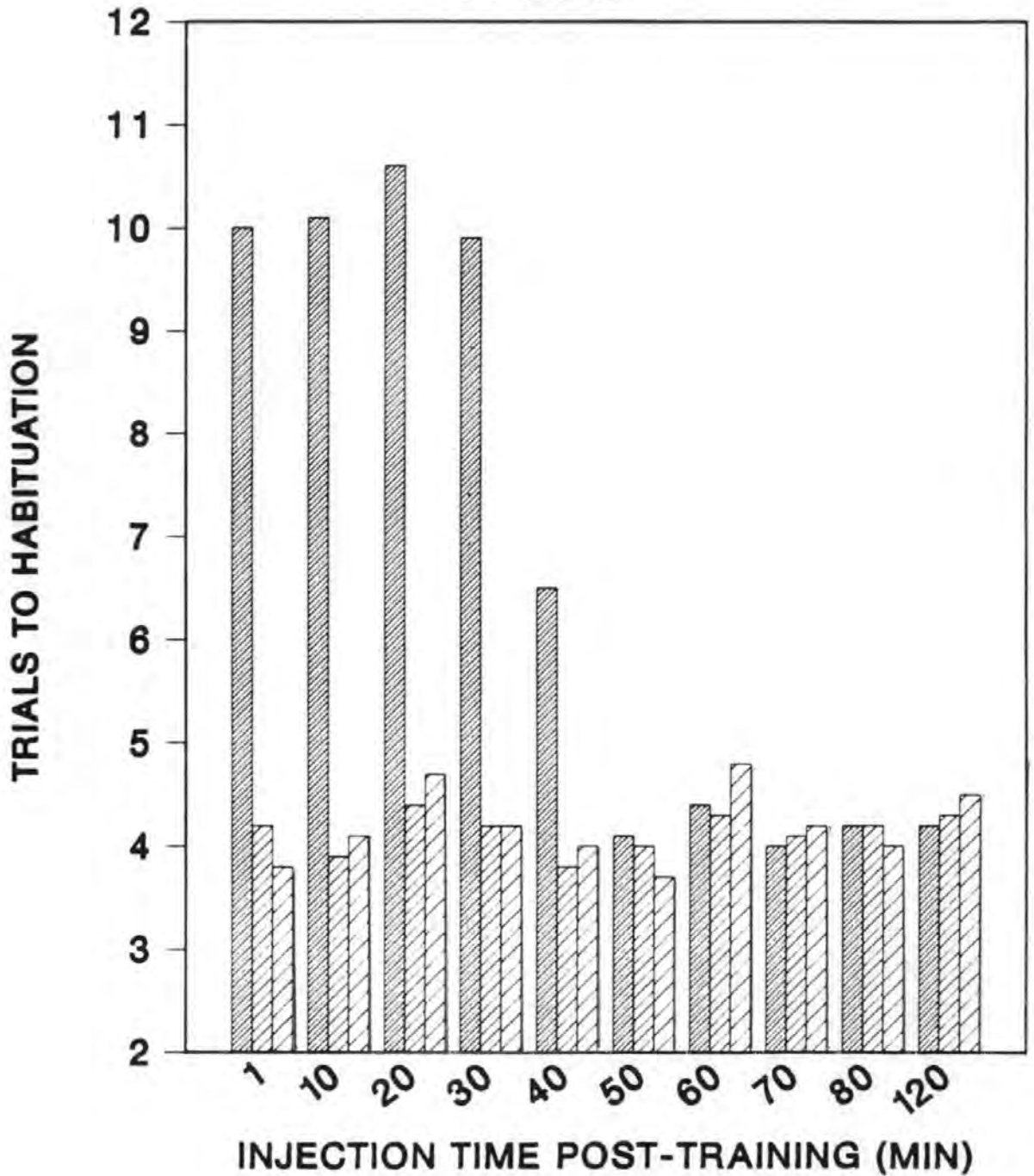


FIGURE 13.3

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE



( $F(1/270) = 1984.610, p < .001$ ). The following interactions also proved significant time x condition ( $F(9/270) = 17.337, p < .001$ ), time x test ( $F(9/270) = 19.277, p < .001$ ), condition x test ( $F(2/270) = 140.977, p < .001$ ), and time x condition x test ( $F(9/270) = 18.501, p < .001$ ).

Analysis of simple main effects showed long term habituation was prevented from drug injections at 1, 10, 20, and 30 mins post-training ( $F's(9/270) < 1$ ). There was no retention in these groups. Injections of puromycin after this had no effect on long term habituation. There was significant retention in groups with post-training injection times of 40, 50, 60, 70, 80, and 120 minutes ( $F(9/270) = 742.94, 734.00, 699.89, 808.99, 799.98, \text{ and } 776.01, p < .001$ ) respectively) (see Figure 13.3).

#### DOSE 2 (25yg/snail)

Analysis in a 3-way ANOVA again revealed a significant effect of time ( $F(9/270) = 6.863, p < .001$ ), condition ( $F(2/270) = 488.523, p < .001$ ), and test ( $F(1/270) = 713.523, p < .001$ ). The following interactions also reached significance time x condition ( $F(9/270) = 5.046, p < .001$ ), time x test ( $F(9/270) = 4.59, p < .05$ ), condition x test ( $F(2/270) = 445.122, p < .001$ ), and time x condition x test ( $F(9/270) = 8.210, p < .001$ ).

Analysis of simple main effects revealed pre-training injection time or condition had no effect on short term habituation (see Figure 13.4) ( $F's(9/270) < 1$ ). Comparisons between training and retest scores revealed that there was



FIGURE 13.4

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 2)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

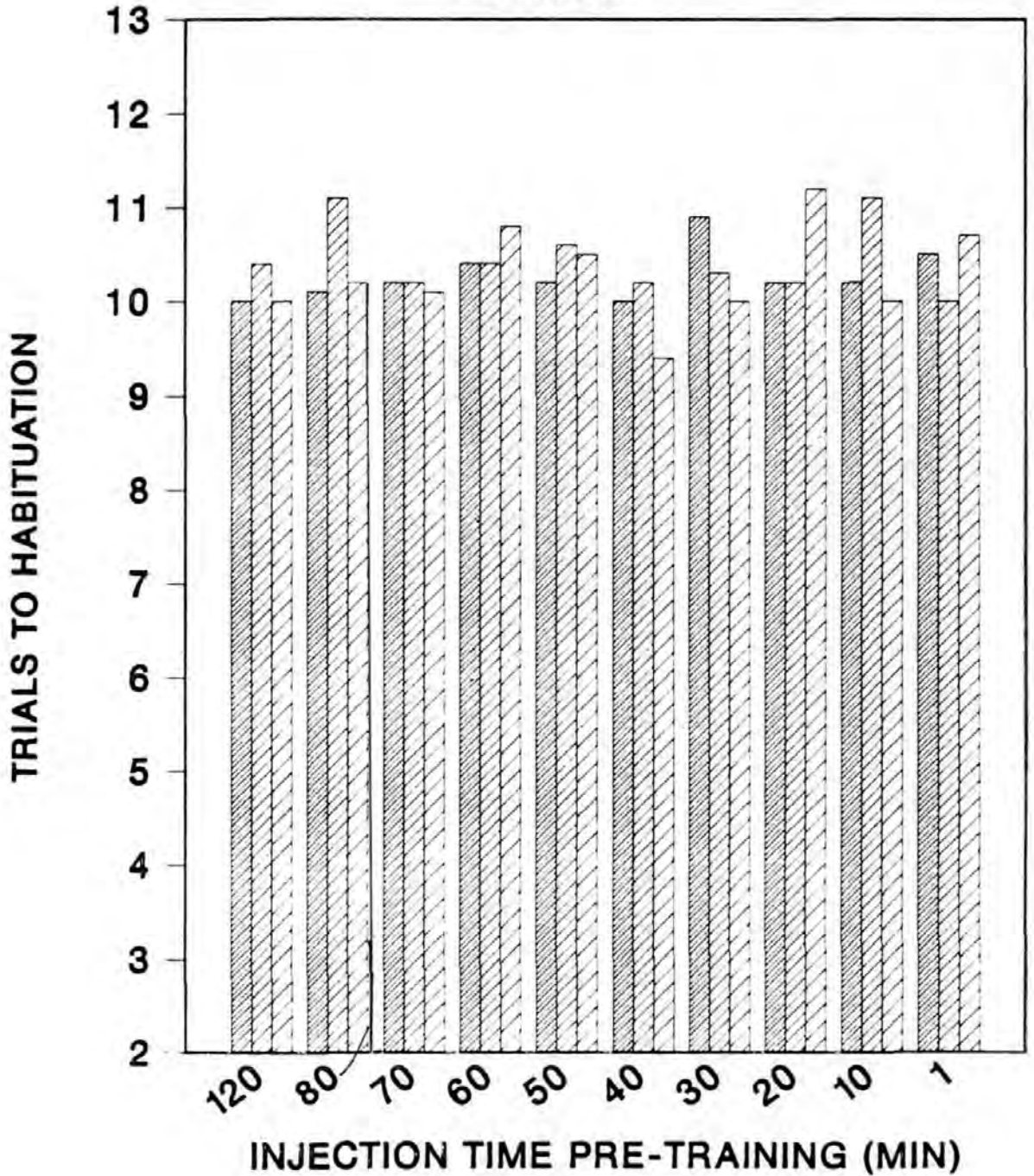


FIGURE 13.5

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

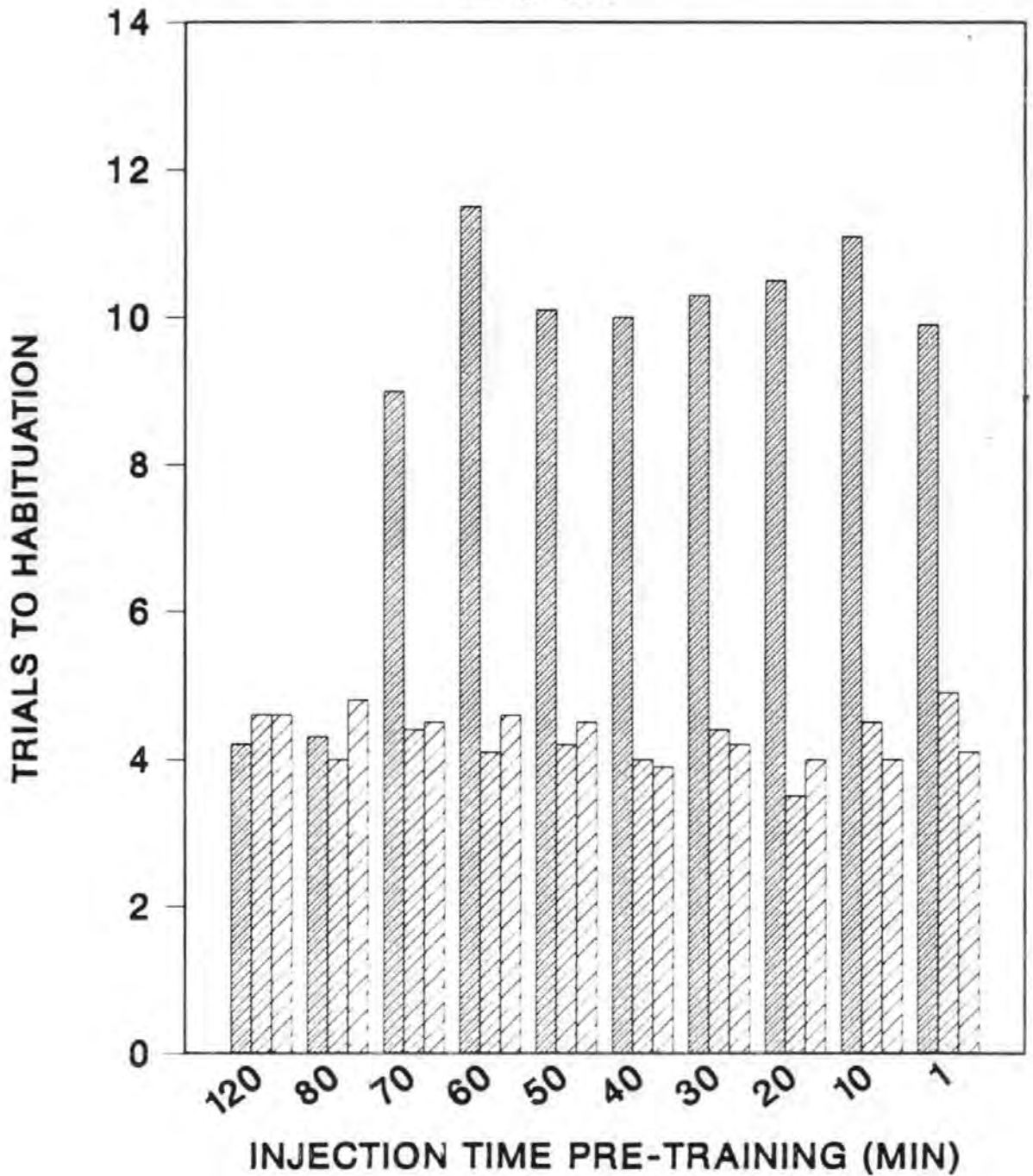
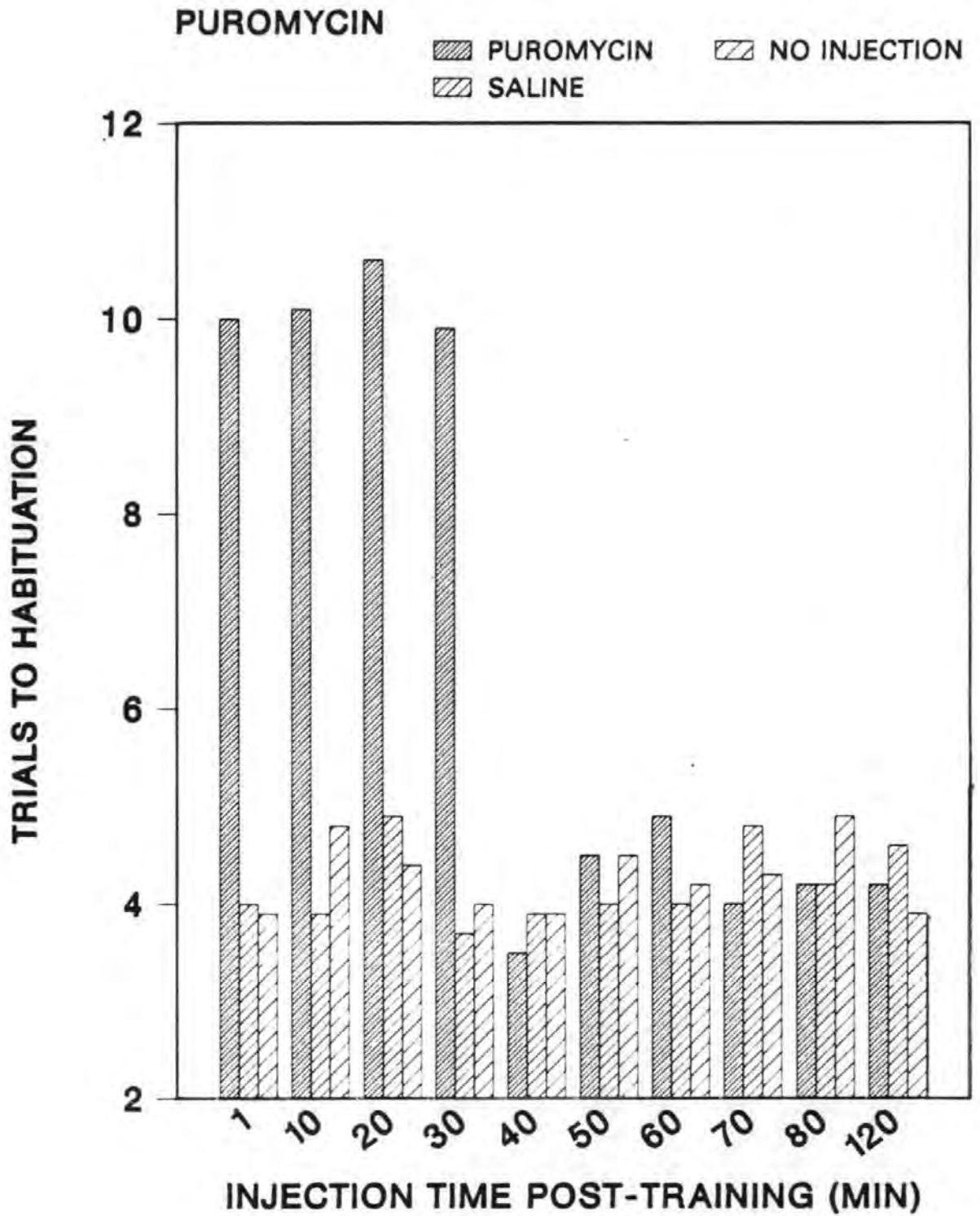


FIGURE 13.6

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)



no long term habituation present in snails that were injected with drug 70 minutes pre-training or in snails injected at any of the other closer pre-training injection times ( $F's(9/270) < 1$ ) (see Figure 13.5). Normal retention was evidenced in groups which received drug 80 or 120 mins prior to training ( $F(9/270) = 789.63$  and  $809.74$ ,  $p < .001$  respectively).

Post-training injection times of puromycin at this dose were also analysed in a 3-way ANOVA and again revealed significant effects of time ( $F(9/270) = 18.063$ ,  $p < .001$ ), condition ( $F(2/270) = 146.062$ ,  $p < .001$ ), and test ( $F(1/270) = 1646.693$ ,  $p < .001$ ). The following interactions also were significant time x condition ( $F(9/270) = 15.729$ ,  $p < .001$ ), time x test ( $F(9/270) = 16.291$ ,  $p < .001$ ), condition x test ( $F(1/270) = 146.062$ ,  $p < .001$ ), and time x condition x test ( $F(9/270) = 16.326$ ,  $p < .001$ ).

Analysis of simple main effects revealed that drug injections up to 30 minutes post-training disrupted long term habituation, there was no difference between training and retest scores in these groups ( $F's(9/270) < 1$ ). Injections after this (40, 50, 60, 70, 80, and 120 min) had no effect on long term habituation ( $F's(9/540) = 598.99$ ,  $671.01$ ,  $583.92$ ,  $577.52$ ,  $600.79$ , and  $633.68$ ,  $p < .001$  respectively) (see Figure 13.6).

#### DOSE 3 250yg/snail

Analysis of pre-training injection times at this dose in a 3-way ANOVA revealed significant effects of time

( $\underline{F}(9/270) = 5.688, p < .001$ ), condition ( $\underline{F}(2/270) = 486.454, p < .001$ ), and test ( $\underline{F}(1/270) = 712.127, p < .001$ ) with the following interactions; time x condition ( $\underline{F}(9/270) = 5.883, p < .001$ ), time x test ( $\underline{F}(9/270) = 3.957, p < .001$ ), condition x test ( $\underline{F}(1/270) = 457.200, p < .001$ ), and time x condition x test ( $\underline{F}(9/270) = 7.361, p < .001$ ).

Analysis of simple main effects revealed, from comparisons of between pharmacological conditions, short term habituation was not disrupted by injection from any of the pre-training intervals ( $\underline{F}'_s(9/270) < 1$ ) (see Figure 13.7). Long term habituation was disrupted by drug injections 80 minutes pre-training and all shorter pre-training injection intervals ( $\underline{F}(9/270) = 348.93, 307.64, 342.79, 299.67, 326.22, 358.63, \text{ and } 356.77, p < .001$  respectively). Injections 120 min prior to training had no effect on long term habituation. Snails in this group showed savings at retest compared to training ( $\underline{F}(9/270) = 729.48, p < .001$ ) (see Figure 13.8).

Analysis of post injection times at this dose in a 3-way ANOVA revealed significant effects of time ( $\underline{F}(9/270) = 7.880, p < .001$ ), condition ( $\underline{F}(2/270) = 582.457, p < .001$ ), and test ( $\underline{F}(1/270) = 512.127, p < .001$ ) with significant interactions of time x condition ( $\underline{F}(9/270) = 10.672, p < .001$ ), time x test ( $\underline{F}(9/270) = 9.756, p < .001$ ), condition x test ( $\underline{F}(1/270) = 500.200, p < .001$ ), and time x condition x test ( $\underline{F}(9/270) = 8.995, p < .001$ ).

Analysis of simple main effects revealed drug injections up to 40 minutes post-training prevented long term habituation ( $\underline{F}'_s(9/270) < 1$ ), and for injections times

FIGURE 13.7

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 3)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

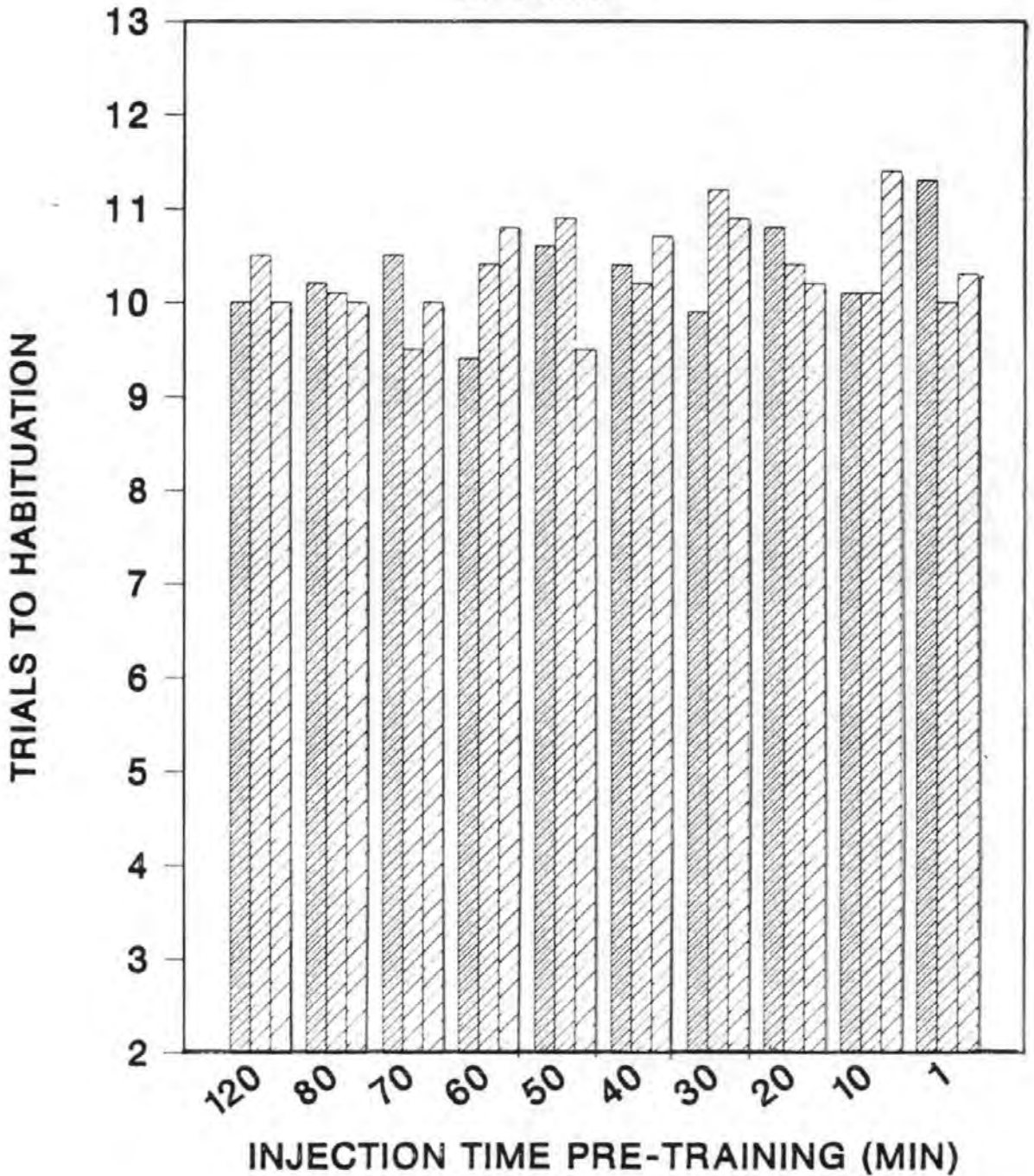


FIGURE 13.8

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)

PUROMYCIN

PUROMYCIN NO INJECTION  
SALINE

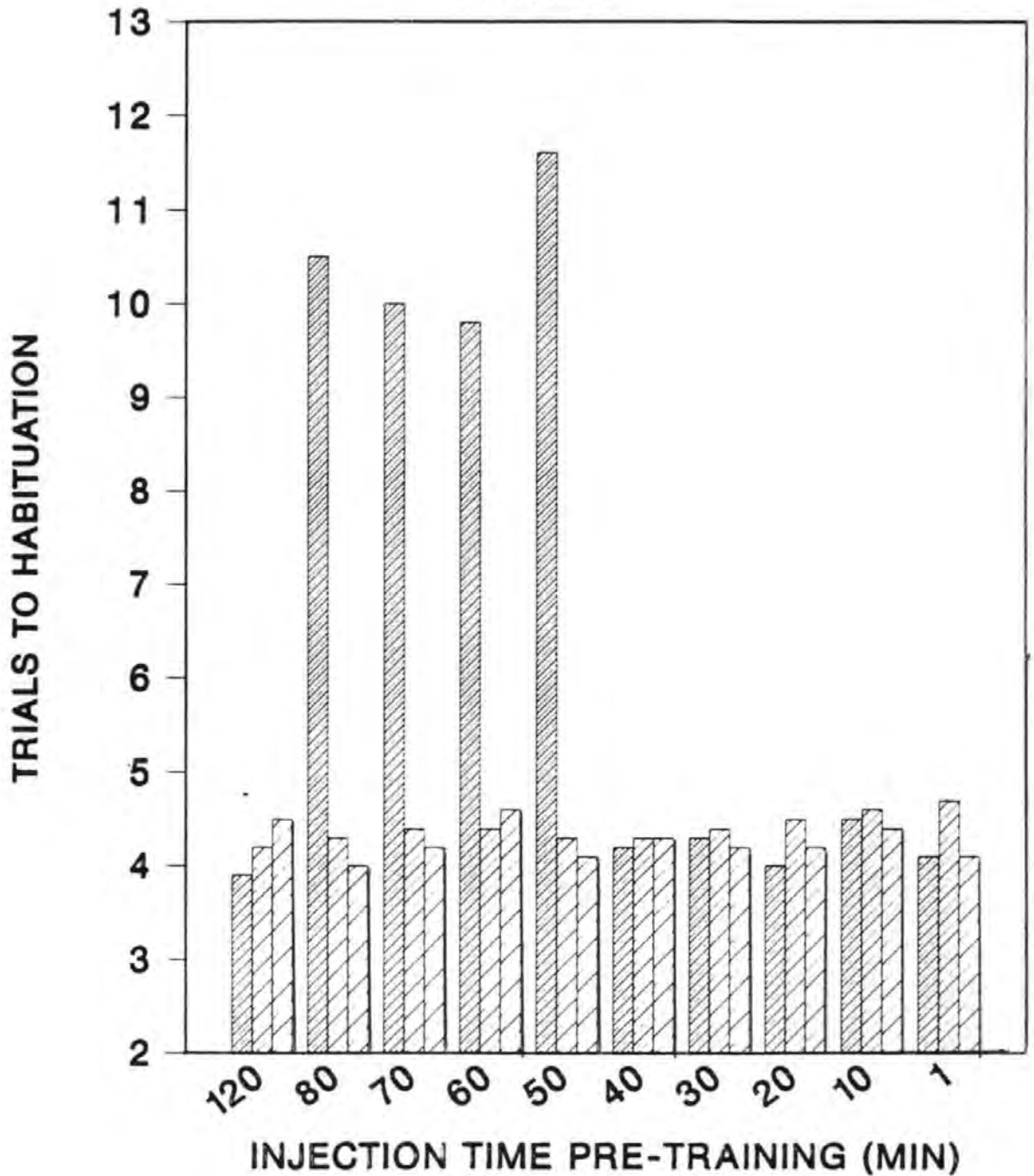


FIGURE 13.9

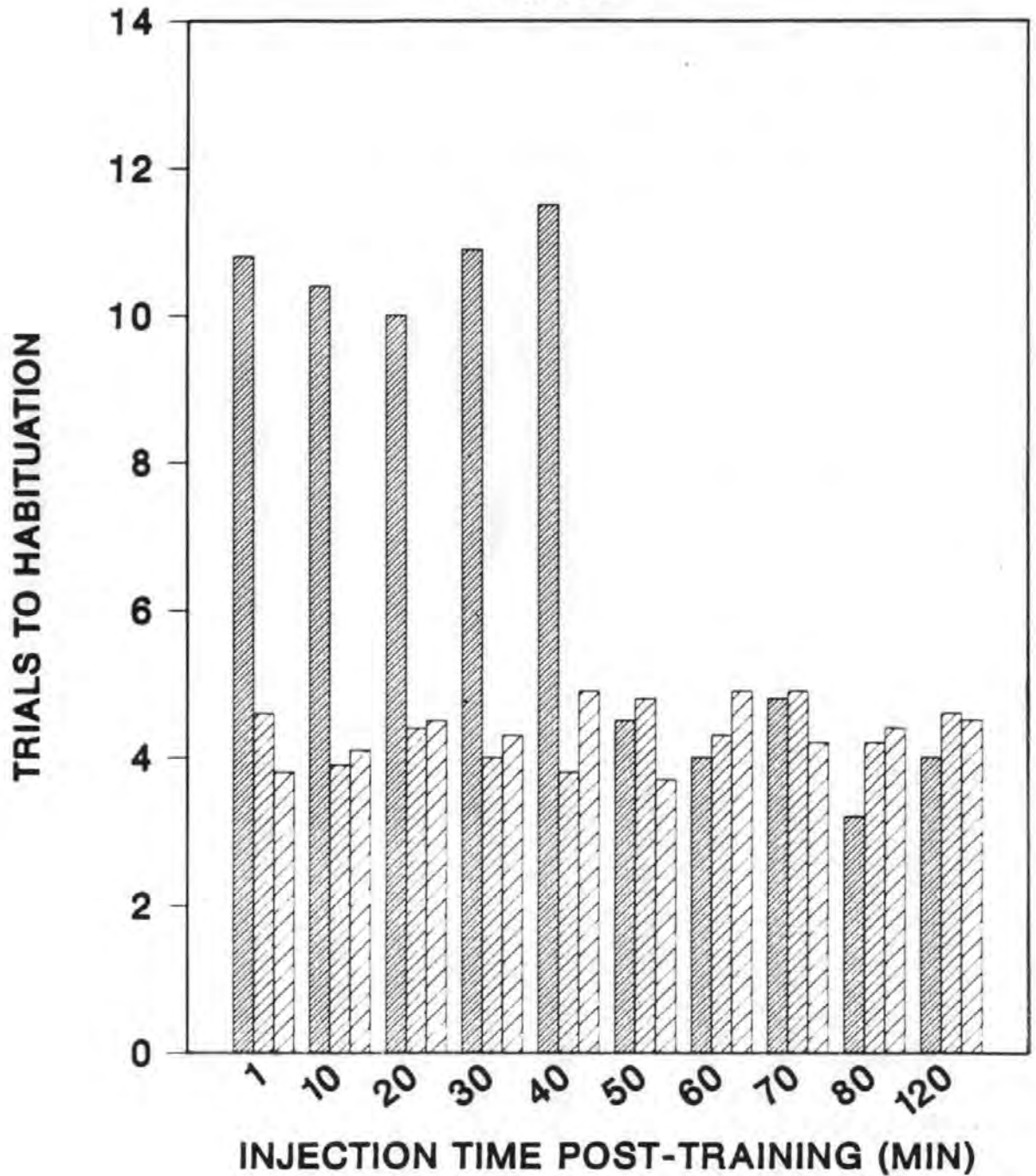
EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)

PUROMYCIN

PUROMYCIN

NO INJECTION

SALINE





after that, the injections of puromycin had no effect, as snails in these groups all showed a saving in trials to habituation in retest ( $F$ 's (9/540) = 499.79, 603.58, 500.09, 483.87, and 545.34,  $p < .001$ , respectively) (see Figure 13.9).

## DISCUSSION

From the above experiment, it was concluded that long term habituation is dependent on protein synthesis in training and in a brief time window post training of between 30 and 40 mins. Inhibition of protein synthesis within this window is sufficient to disrupt the long term process. Further, dose of Puromycin affects the time the drug can cause the effect pre-training, but it did not appear to have any major effect on post-training interval. This suggests a critical period of protein synthesis. By comparing groups, the drug appears to remain active, in terms of its behavioural effect, for between 40 and 80 mins dependent on dose, and this corresponds to results from biochemical analysis (see Chapter 7).

## EXPERIMENT 2

### TIME/EFFECT ANALYSIS FOR THREE DOSES OF ACTINOMYCIN D

The above experiment was repeated for the antibiotic Actinomycin D. The results are depicted in Figure 13.10 to 13.18 and, again show clear effects of time of injection on disruption of long term habituation. This experiment was

conducted on the nights of April 1 to the 28, 1986, between 1600 and 1030. Temperature in the laboratory varied from 18 to 20 C. Within each dose, pre-training injection groups are analysed initially, followed by post-training injection times.

#### DOSE 1 (2.5yg/snail)

Experiment 2 was analysed in an identical way to Experiment 1. A 3-way ANOVA revealed significant effects of time ( $F(9/270) = 20.254, p < .001$ ), condition ( $F(2/270) = 128.930, p < .001$ ), and test ( $F(1/270) = 1007.221, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 14.970, p < .001$ ), time x test ( $F(9/270) = 15.008, p < .001$ ), condition x test ( $F(2/270) = 164.675, p < .001$ ), and time x condition x test ( $F(9/270) = 13.529, p < .001$ ).

Analysis of simple main effects revealed no effect of any of the pre-training drug injections on short term habituation compared to either saline or no injection snails ( $F'S(9/270) < 1$ ) (see Figure 13.10). Long term habituation was not demonstrated by snails that received injection of Actinomycin D at 1, 10, 20, 30, 40, 50, 60, or 70 mins pre-training. There was no difference in trials to habituation between training and retest ( $F(9/540) < 1$ ) (see Figure 10.10 and 10.11). Normal retention was evidenced by drug groups injected 80 and 120 mins pre-training ( $F(9/540) = 497.96, \text{ and } 571.582, p < .001$  respectively) (see Figure 13.11).

Analysis of post-training injection groups in a 3-way

FIGURE 13.10

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 1)

ACTINOMYCIN D

ACTINOMYCIN D NO INJECTION  
SALINE

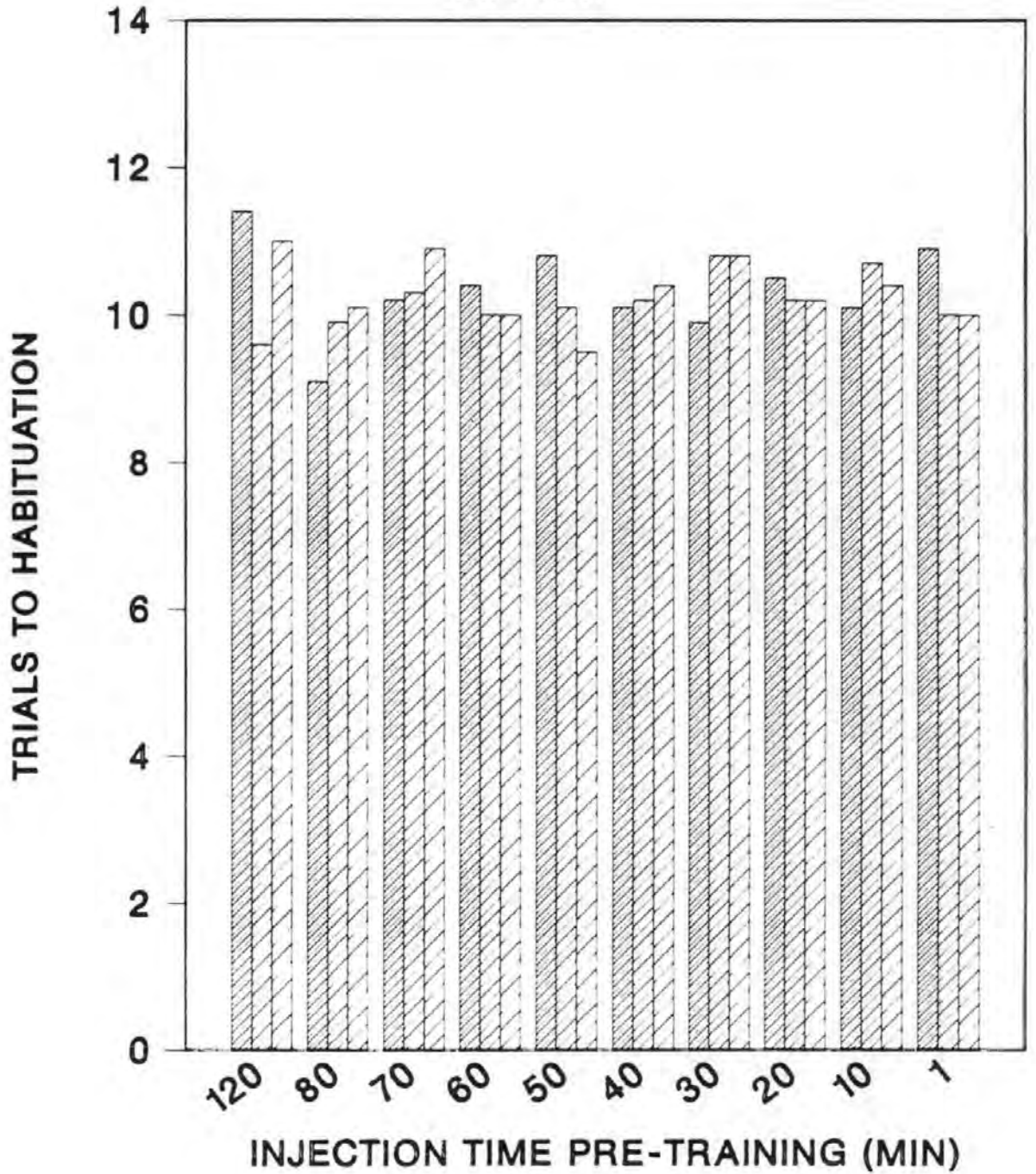


FIGURE 13.11

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)

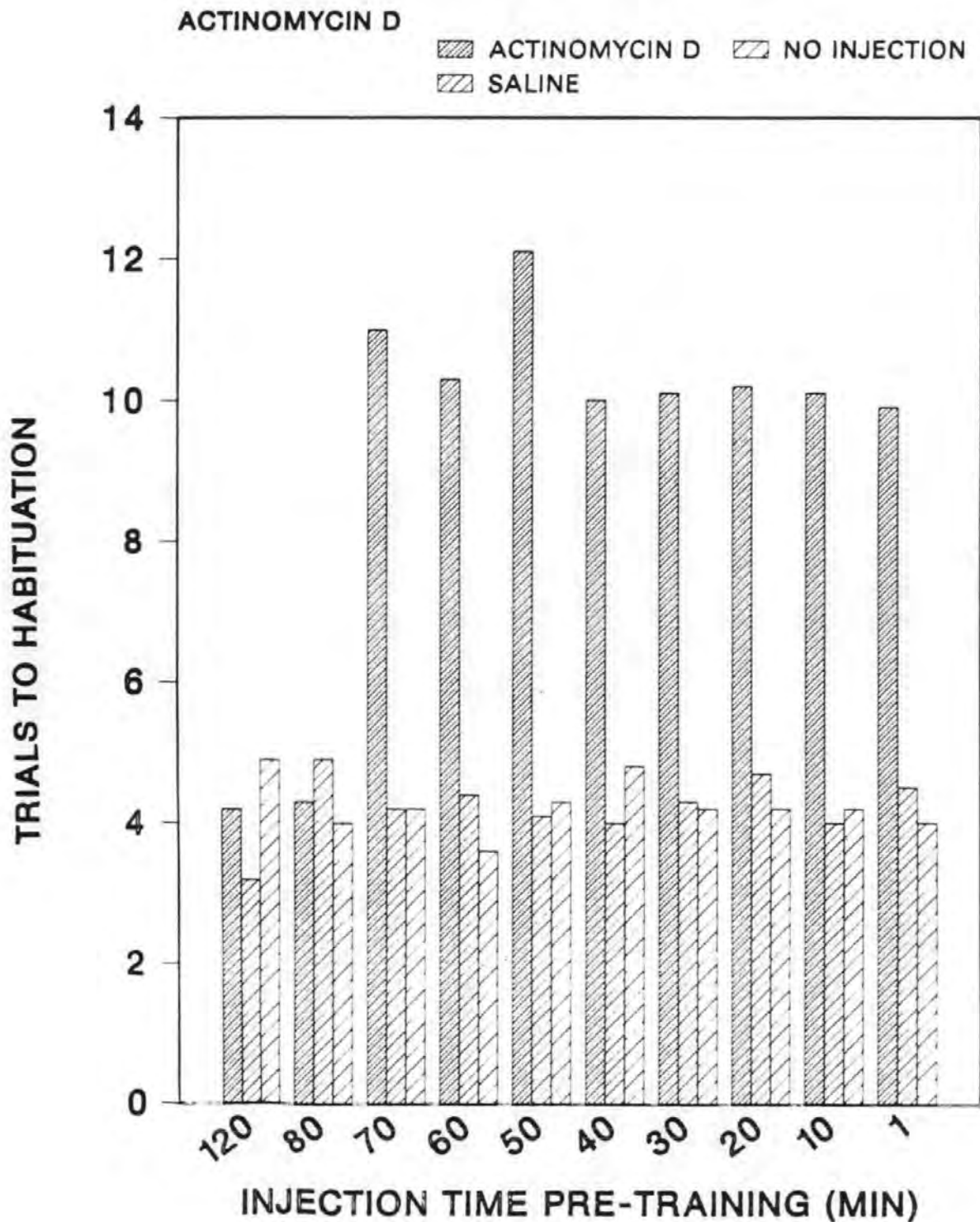
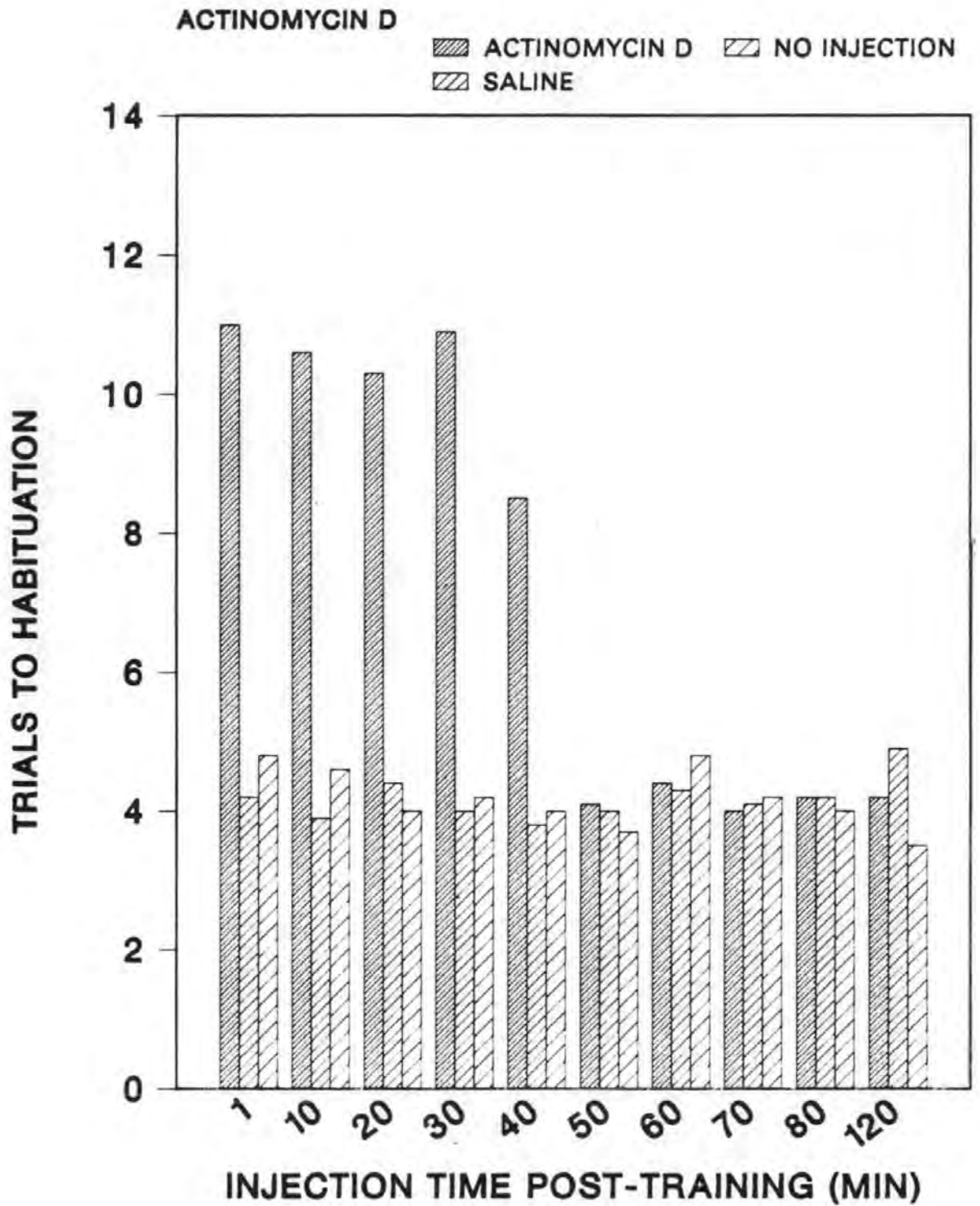


FIGURE 13.12

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)



ANOVA revealed significant effects of time ( $F(9/270) = 13.729, p < .001$ ), condition ( $F(2/270) = 165.827, p < .001$ ), test ( $F(1/270) = 1341.819, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 17.276, p < .001$ ), time x test ( $F(9/270) = 16.093, p < .001$ ), condition x test ( $F(2/270) = 128.999, p < .001$ ), and time x condition x test ( $F's(2/270) = 18.741, p < .001$ ).

Analysis of simple main effects showed long term habituation was disrupted if the drug was injected up to 40 mins post-training. No retention was evidenced in these groups ( $F(9/270) < 1$ ). After this time, the actinomycin D had no effect on long term habituation in that snails showed normal retention of the learning in these groups ( $F's(9/270) = 749.372, 763.589, 699.998, 791.258, \text{ and } 687.997, p < .001$  respectively) (see Figure 13.12).

#### DOSE 2 (2.5yg/snail)

A 3-way ANOVA revealed significant effects of time ( $F(9/270) = 13.419, p < .001$ ), condition ( $F(2/270) = 127.959, p < .001$ ), and test ( $F(1/270) = 1548.002, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 13.451, p < .001$ ), time x test ( $F(9/270) = 15.009, p < .001$ ), condition x test ( $F(2/270) = 141.847, p < .001$ ), and time x condition x test ( $F(9/270) = 14.803, p < .001$ ).

From analysis of simple main effects, no effect on short term habituation was found from any pre-training injection protocol ( $F(9/270) < 1$ ) (see Figure 13.13), but long term habituation was disrupted by drug injections times

FIGURE 13.13

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 2)

ACTINOMYCIN D

ACTINOMYCIN D NO INJECTION  
SALINE

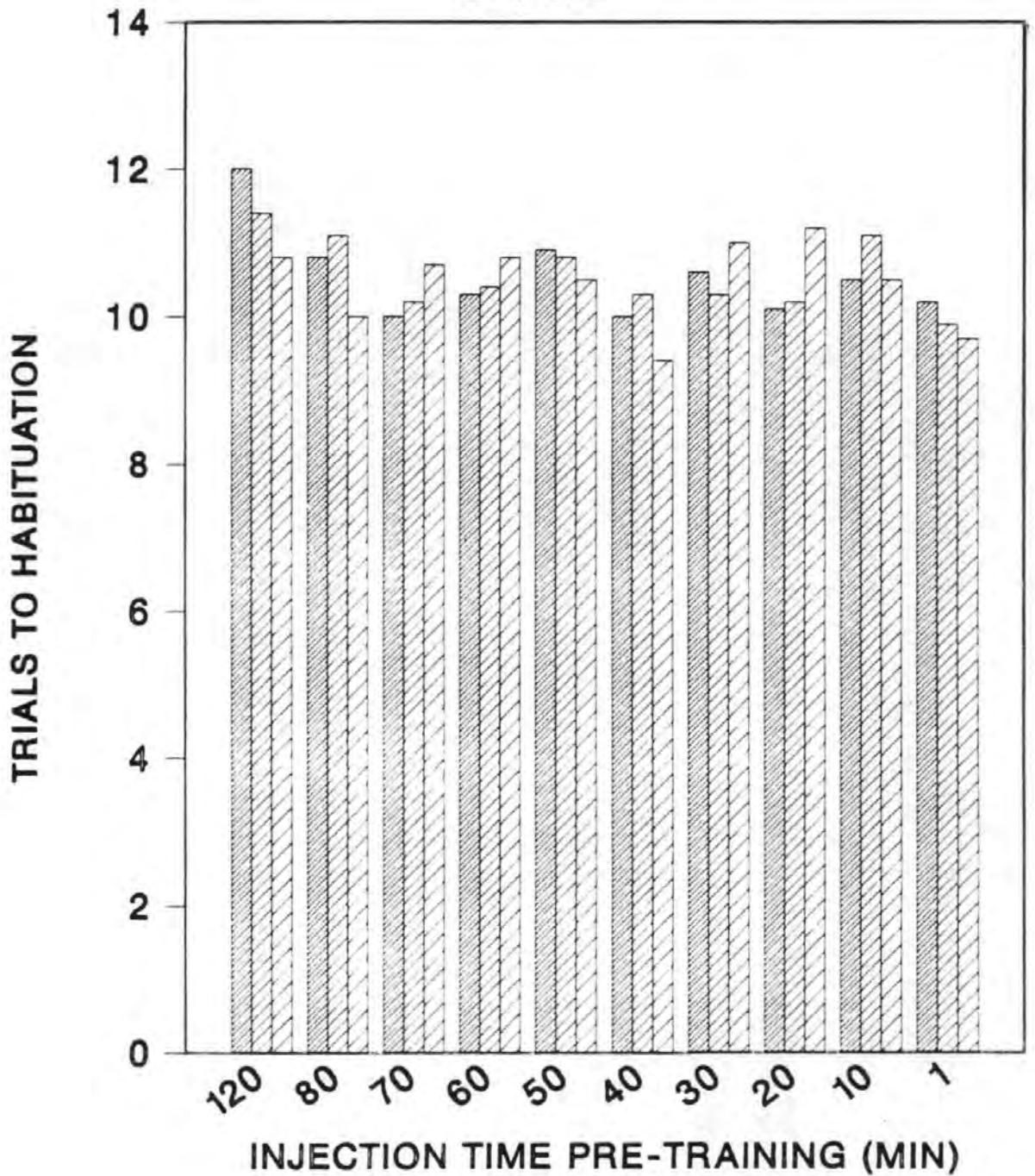


FIGURE 13.14

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)

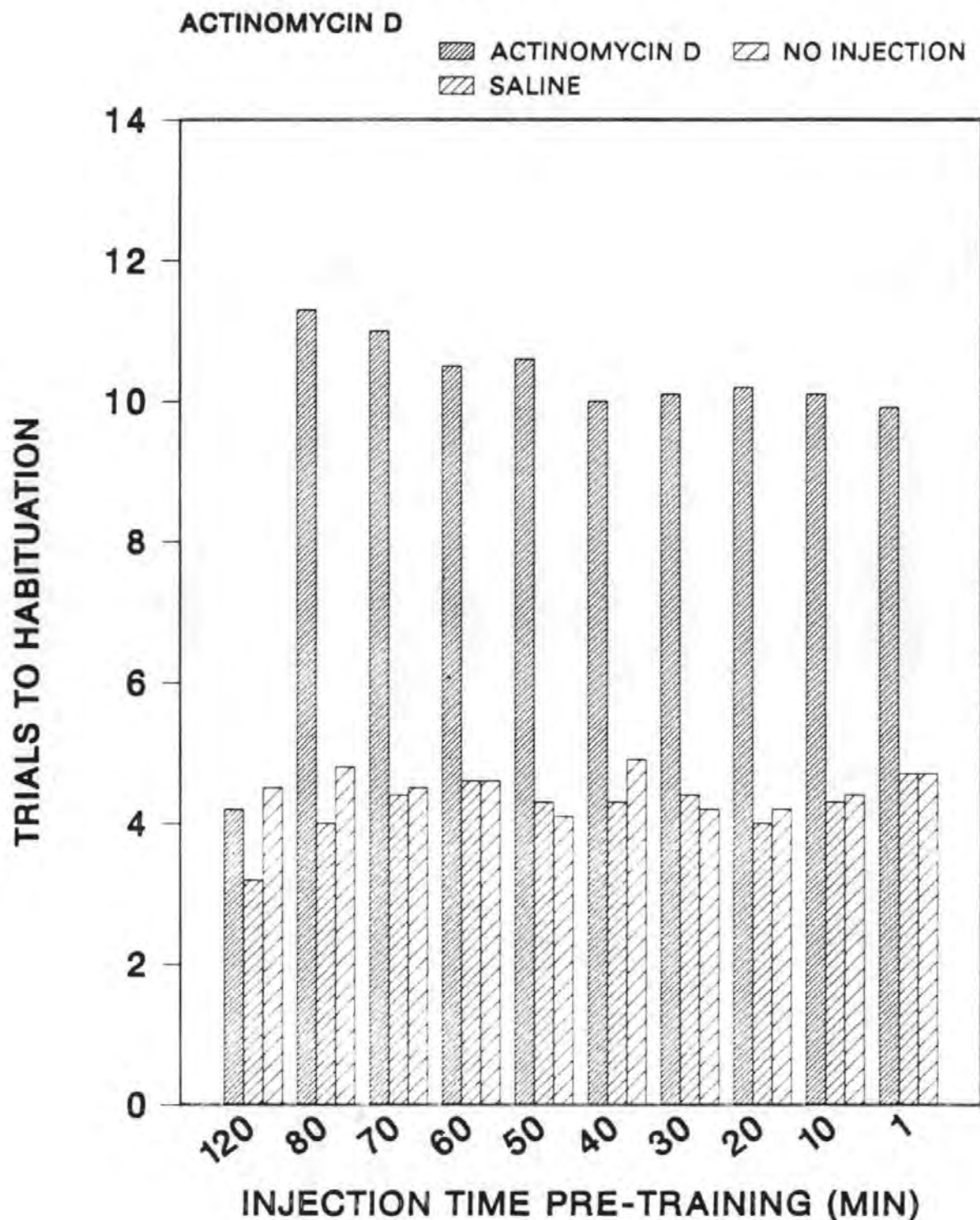
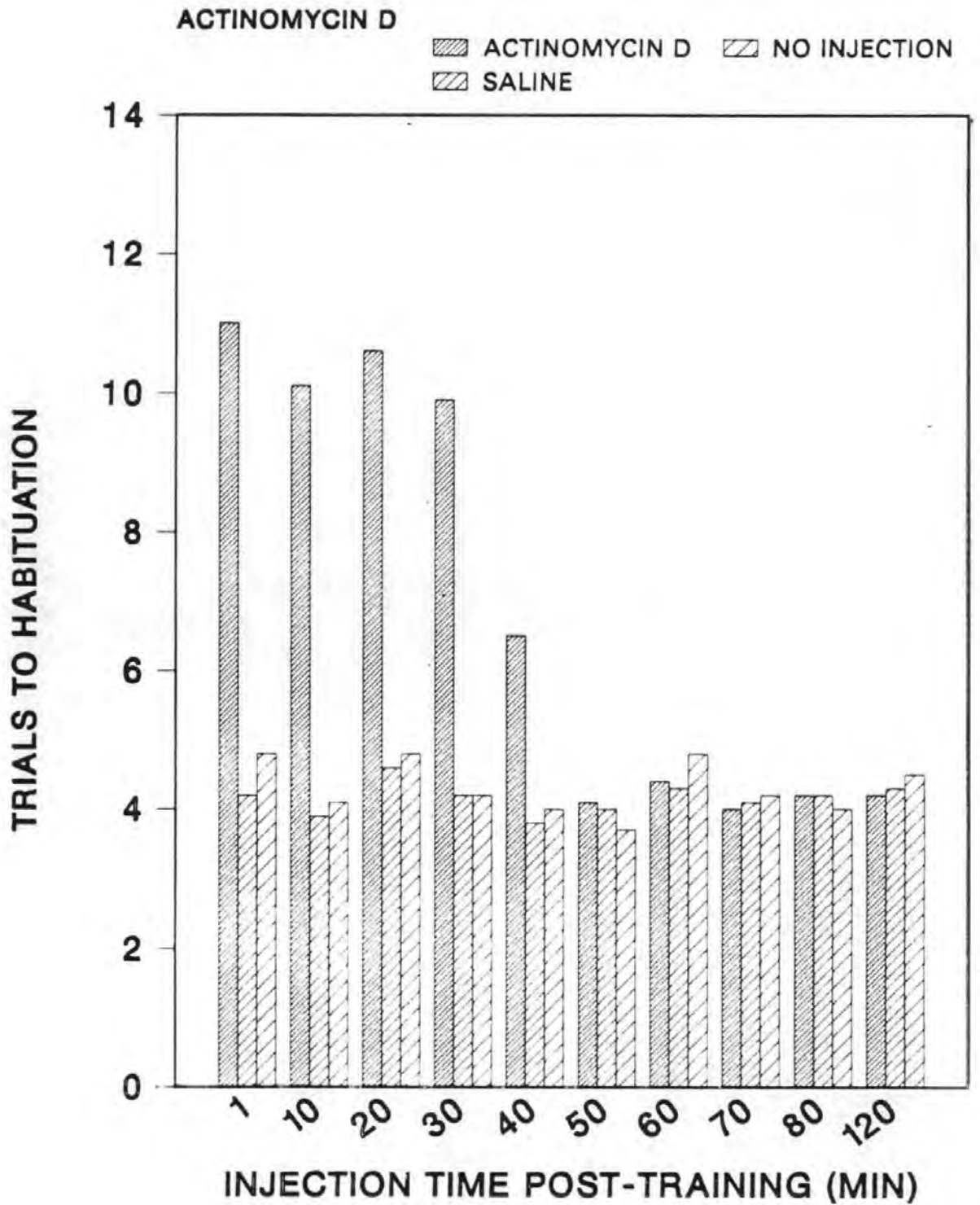




FIGURE 13.15

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)



up to and including 80 minutes pre-training. Comparisons of training and retest trials to habituation in these groups revealed no significant difference ( $F(9/270) < 1$ ). However, snails that received drug 120 minutes before training showed normal retention ( $F(9/540) = 758.352, p < .001$ ) (see Figure 13.14)

For post-training injection times, a 3-way ANOVA showed significant effects of time ( $F(9/270) = 20.078, p < .001$ ), condition ( $F(2/270) = 149.997, p < .001$ ), and test ( $F(1/270) = 1008.347, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 19.344, p < .001$ ), time x test ( $F(9/270) = 20.726, p < .001$ ), condition x test ( $F(2/270) = 160.496, p < .001$ ), and time x condition x test ( $F(9/270) = 23.927, p < .001$ ).

Analysis of simple main effects revealed the drug at this dose disrupted long term habituation when injected up to and including 40 minutes post-training, as comparisons of training and retest scores in these groups showed no saving in trials to habituation between training and retest ( $F(9/270) < 1$ ). A saving was evident in later drug injection post-training time of 50, 60, 70, 80, and 120 minutes ( $F(9/270) = 208.68, 234.85, 219.59, 233.27, \text{ and } 239.81, p < .001$  respectively). (see Figure 13.15).

#### DOSE 3 (25yg/snail)

Analysis of the pre-training injection groups in a 3-way ANOVA revealed a significant effect of time ( $F(9/270) = 14.759, p < .001$ ), condition ( $F(2/270) = 151.654, p < .001$ ),

and test ( $F(1/270) = 998.989, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 15.443, p < .001$ ), time x test ( $F(9/270) = 16.001, p < .001$ ), condition x test ( $F(2/270) = 135.482, p < .001$ ), and time x condition x test ( $F(9/270) = 13.338, p < .001$ )

From analysis of simple main effects, actinomycin D at this dose again showed no effect on short term habituation for any of the pre-training drug injection times. Comparisons of trials to habituation between the three treatments showed no significant effect ( $F(9/270) < 1$ ) (see Figure 13.16). Long term habituation was disrupted by injections up to and including 80 minutes pre-training (see Figure 13.17). Snails in these groups showed no savings in trials to habituation at retest ( $F(9/270) < 1$ ). Injection of drug 120 minutes pre training had no effect on long term habituation, snails showed significant retention ( $F(9/270) = 619.007, p < .001$ ).

Analysis of post-training injection times in a 3-way ANOVA revealed significant effects of time ( $F(9/270) = 19.083, p < .001$ ), condition ( $F(2/270) = 164.662, p < .001$ ), and test ( $F(1/270) = 1403.937, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 18.445, p < .001$ ), time x test ( $F(9/270) = 19.438, p < .001$ ), condition x test ( $F(2/270) = 131.597, p < .001$ ), and time x condition x test ( $F(9/270) = 17.993, p < .001$ ).

Simple main effect analysis revealed that from, post-training injections of the drug up to and including 30 mins, long term habituation was disrupted in that there was no difference between training score and retest score

FIGURE 13.16

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 3)

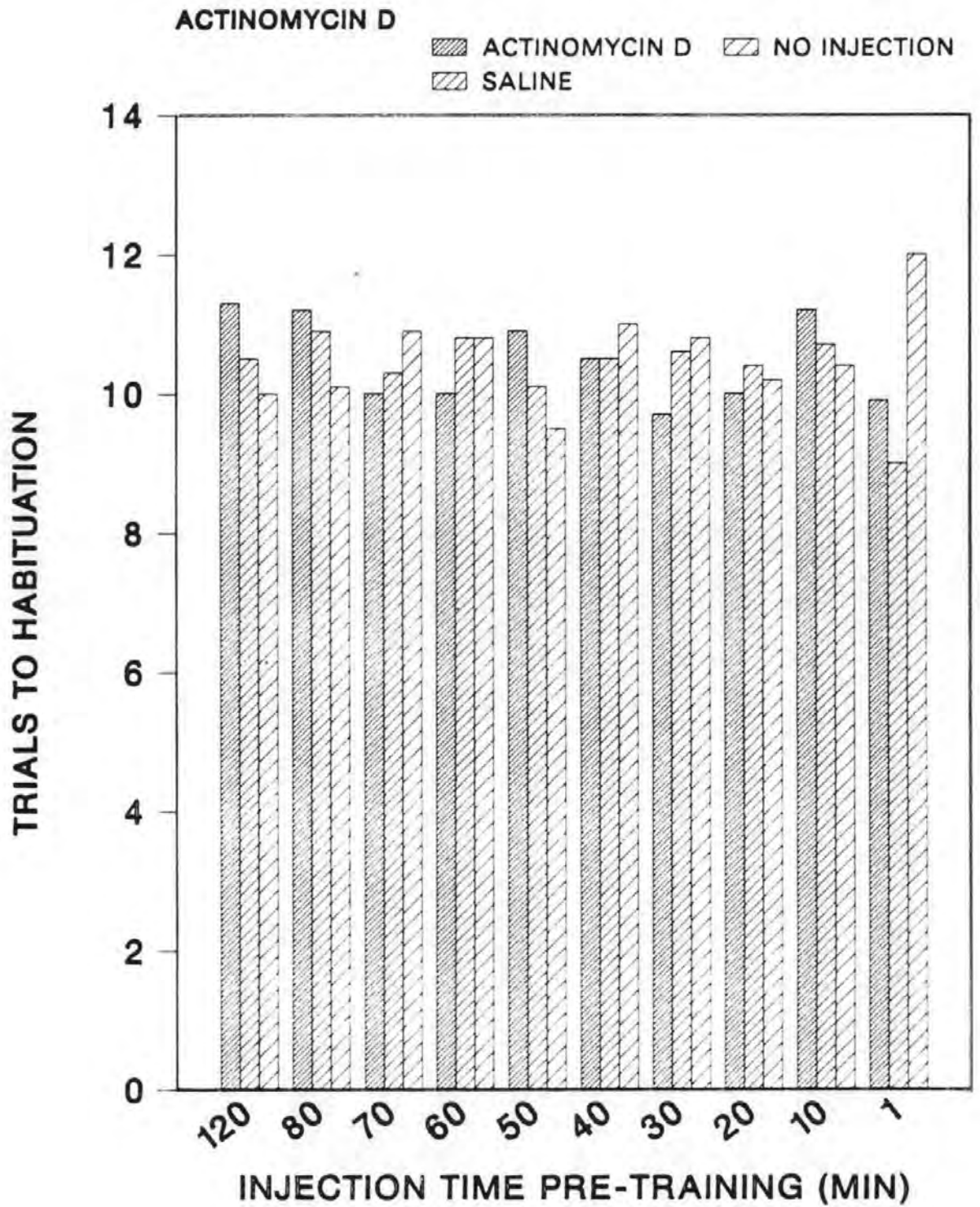


FIGURE 13.17

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)

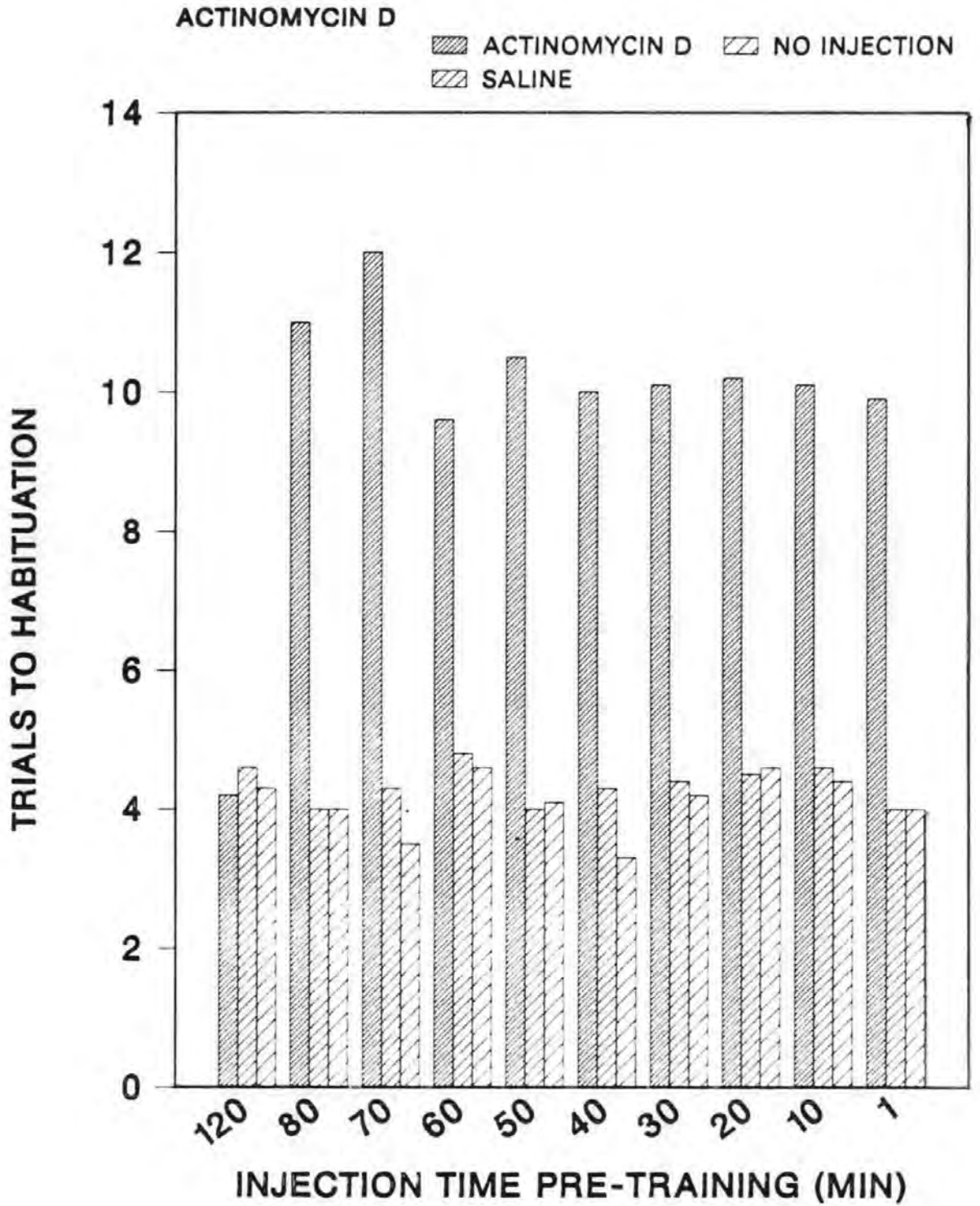
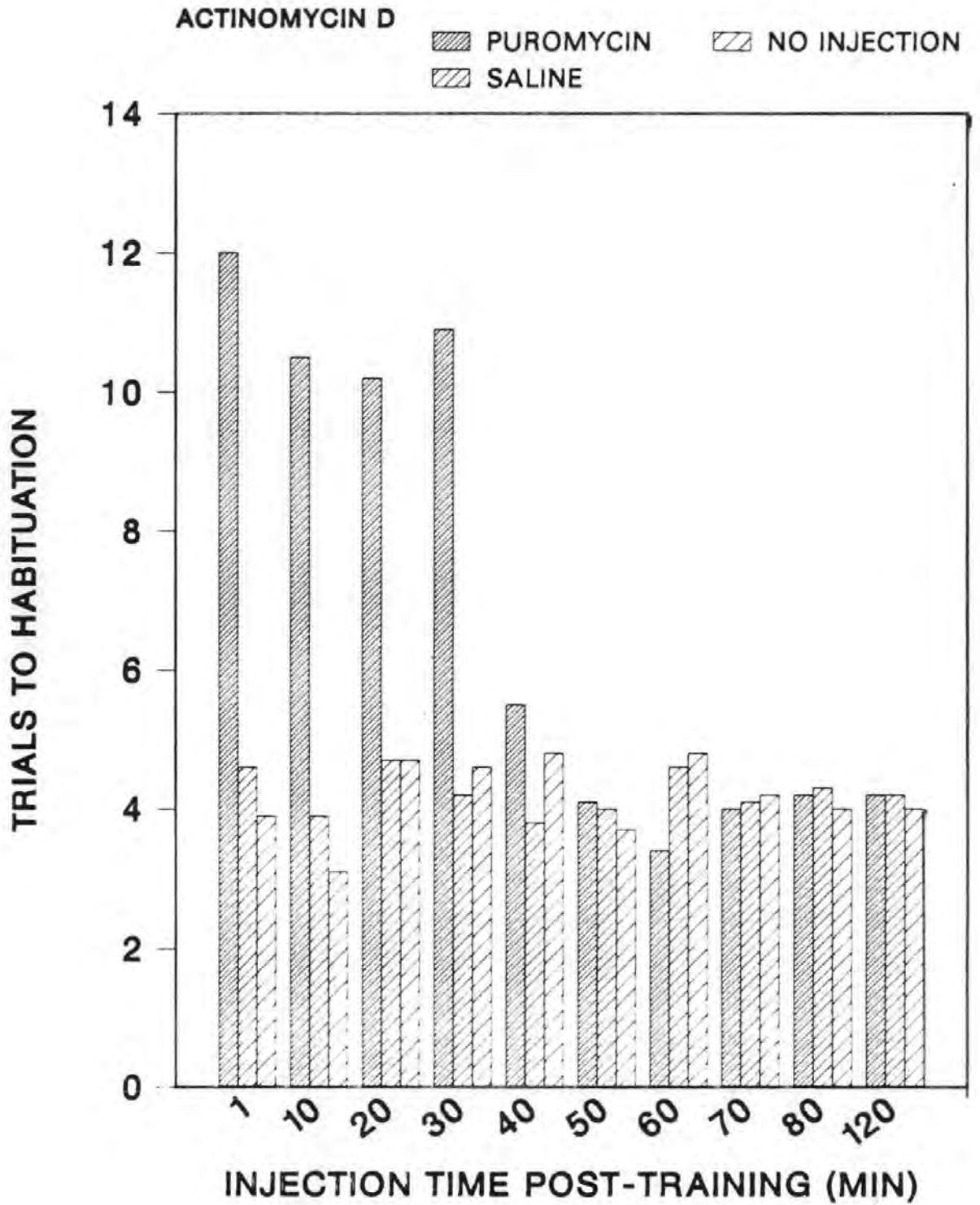


FIGURE 13.18

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)



( $F(9/270) < 1$ ) (see Figure 13.18). Outside this time window, there was no amnesic effect of the actinomycin D, all groups showed significant retention in retest ( $F(9/540) = 398.96, 541.829, 499.721, 409.132, 487.354, \text{ and } 500.872, p < .001$  respectively).

## DISCUSSION.

Actinomycin D could prevent long term habituation at all doses when the drug was active in either training or in a brief period after training. The results of this experiment suggest that actinomycin D reversibly inhibits protein synthesis from for approximately 80 minutes, and, from the 1 minute post-training results, it would appear to initiate protein synthesis inhibition rapidly after injection as the data in Chapter 7 suggests. The sensitive time window for the observed amnesic effect on long term habituation appeared to vary as a function of dose. Injections of drug at intervals greater than 40 minutes after training had no effect on subsequent long term habituation.

## EXPERIMENT 3

### TIME OF EFFECT ANALYSIS FOR ANISOMYCIN AT THREE DOSES

The above experiment was replicated for the antibiotic anisomycin. Results are reported in Figures 13.19 to 13.26 and show a clear effect of time of injection on disruption

of long term habituation. The experiment was conducted on the nights of January 2 to 30, 1986 between 1600 and 1130 temperature in the laboratory varied from 18 to 19 C. At each dose, the 10 pre-training injection conditions were analysed initially, followed by the 10 post-training. At each time the 10 drug treated snails were compared to saline and no injection controls.

#### DOSE 1 (2.5yg/snail)

Analysis of the ten pre-training injection times was in a 3-way ANOVA with between subject factors of time (injection time), and condition (drug, saline or no injection), and a within subject factor of test (training/retest). This revealed a significant effect of time ( $F(9/270) = 13.94, p < .001$ ), condition ( $F(2/270) = 171.165, p < .001$ ), and test ( $F(1/270) = 1437.624, p < .001$ ). There were significant interactions of time x condition ( $F(9/270) = 14.775, p < .001$ ), time x test ( $F(9/270) = 16.380, p < .001$ ), condition x test ( $F(2/270) = 113.701, p < .001$ ), and time x condition x test ( $F(9/270) = 17.057, p < .001$ ).

By comparisons of trials to habituation between conditions, analysis of simple main effects showed anisomycin at this dose had no effect on short term habituation ( $F(9/270) < 1$ ) from any pre-training injection time (see Figure 13.19). Long term habituation was, however, absent in snails that received the drug at 40, 30, 20, 10, and 1 minute pre-training. There was no difference in trials to habituation between training and retest ( $F(9/270) < 1$ ) in



FIGURE 13.19

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 1)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

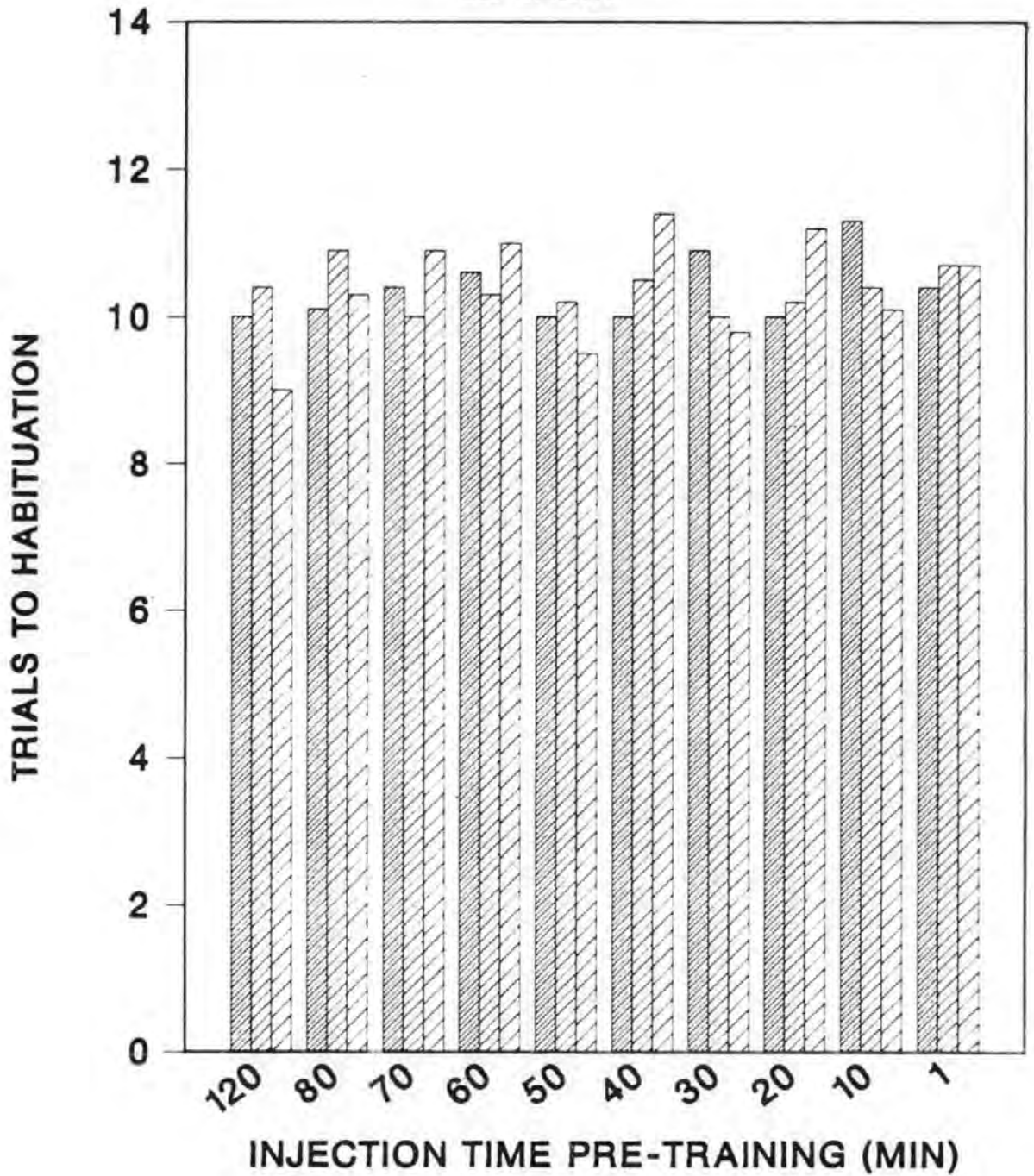


FIGURE 13.20

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

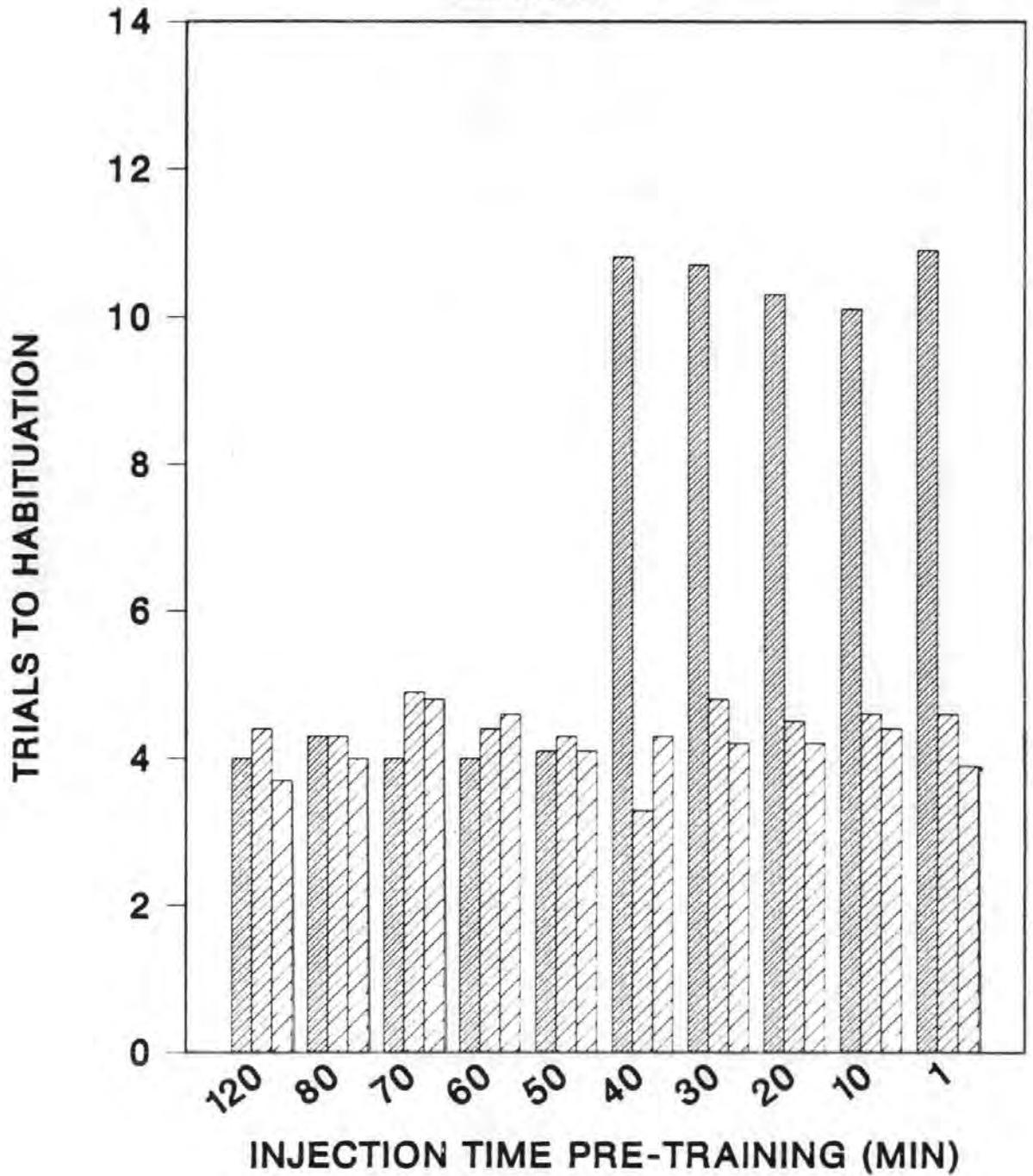
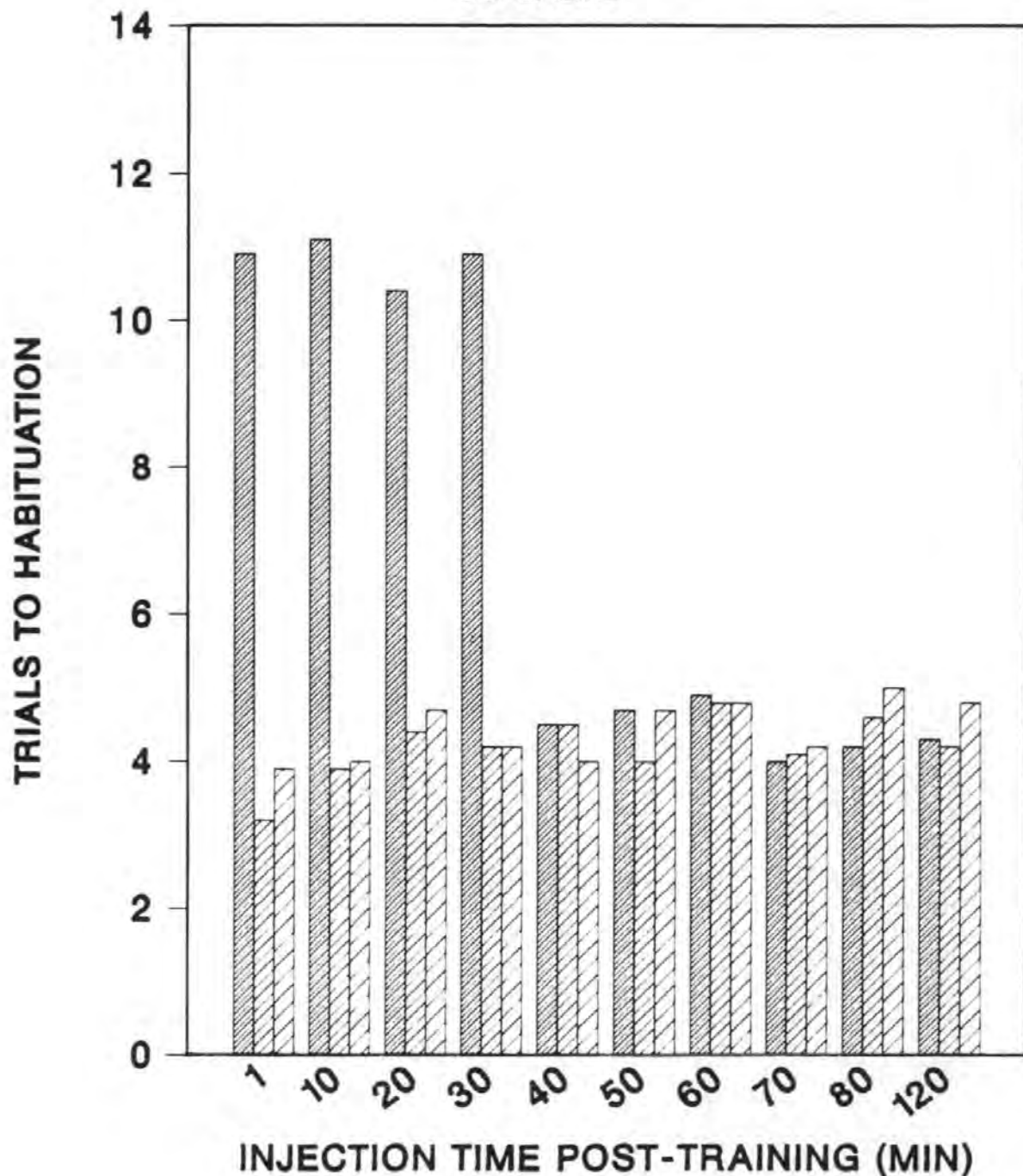


FIGURE 13.21

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 1)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE



these groups. (see Figure 13.20). Earlier injection times (120, 80, 70, 60, and 50 minutes pre-training) had no effect on long term habituation. Snails in these groups showed a saving in trials to habituation compared to retest ( $\underline{F}(9/270) = 675.92, 862.87, 709.42, 833.52, \text{ and } 696.54, p < .001$  respectively).

For the post-training injection groups a 3-way ANOVA showed significant effects of time ( $\underline{F}(9/270) = 18.638, p < .001$ ), condition ( $\underline{F}(2/270) = 159.682, p < .001$ ), and test ( $\underline{F}(1/270) = 1379.560, p < .001$ ). The following interactions also proved significant time x condition ( $\underline{F}(9/270) = 19.084, p < .001$ ), time x test ( $\underline{F}(9/270) = 18.777, p < .001$ ), condition x test ( $\underline{F}(2/270) = 159.892, p < .001$ ), and time x condition x test ( $\underline{F}(9/270) = 16.851, p < .001$ ).

Analysis of simple main effects showed long term habituation was prevented from drug injections at 1, 10, 20, and 30 mins post-training ( $\underline{F}'s(9/270) < 1$ ). Injections of anisomycin after this had no effect on long term habituation. There was significant retention in these groups ( $\underline{F}(9/270) = 766.34, 724.99, 709.89, 799.09, 738.26, \text{ and } 798.91, p < .001$  respectively) (see Figure 13.21).

#### DOSE 2 (25yg/snail)

Analysis in a 3-way ANOVA again revealed a significant effect of time ( $\underline{F}(9/270) = 15.453, p < .001$ ), condition ( $\underline{F}(2/270) = 258.238, p < .001$ ), and test ( $\underline{F}(1/270) = 993.128, p < .001$ ). The following interactions also reached significance time x condition ( $\underline{F}(9/270) = 14.749, p < .001$ ),

time x test ( $F(9/270) = 14.590, p < .05$ ), condition x test ( $F(2/270) = 649.932, p < .001$ ), and time x condition x test ( $F(9/270) = 8.431, p < .001$ ).

Analysis of simple main effects revealed that the drug had no effect on short term habituation at any of the pre-training injection times (see Figure 13.22) ( $F's(9/270) < 1$ ). There was no long term habituation present in snails that were injected 70 mins pre-training or in snails injected at any of the other closer pre-training times ( $F's(9/270) < 1$ ) (see Figure 13.23). Normal retention was evidenced in groups which received drug 80 or 120 minutes prior to training ( $F(9/270) = 774.627$  and  $866.751, p < .001$  respectively).

Post-training injection times of puromycin at this dose were also analysed in a 3 way ANOVA and revealed significant effects of time ( $F(9/270) = 15.963, p < .001$ ), condition ( $F(2/270) = 126.942, p < .001$ ), and test ( $F(1/270) = 1336.013, p < .001$ ). The following interactions were significant, time x condition ( $F(9/270) = 13.798, p < .001$ ), time x test ( $F(9/270) = 15.673, p < .001$ ), condition x test ( $F(1/270) = 129.994, p < .001$ ), and time x condition x test ( $F(9/270) = 16.452, p < .001$ ).

Analysis of simple main effects revealed that drug injections up to 30 minutes post training disrupted long term habituation. There was no difference between training and retest scores in these groups ( $F's(9/270) < 1$ ). Drug injections after this had no effect on long term habituation ( $F's(9/270) = 591.00, 642.937, 584.734, 522.611, 623.71, \text{ and } 611.99, p < .001$ ) (see Figure 13.24).

FIGURE 13.22

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 2)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

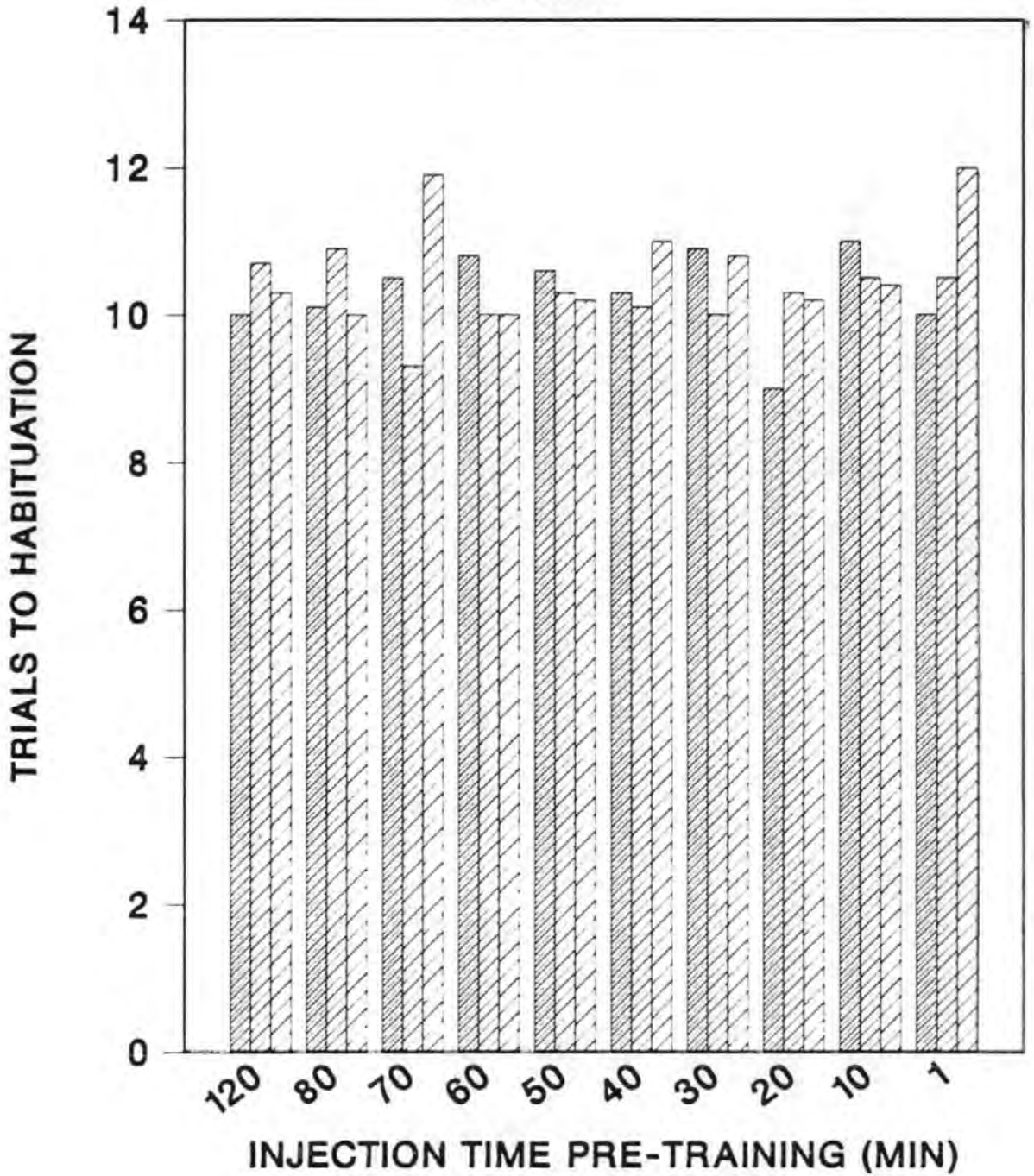


FIGURE 13.23

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

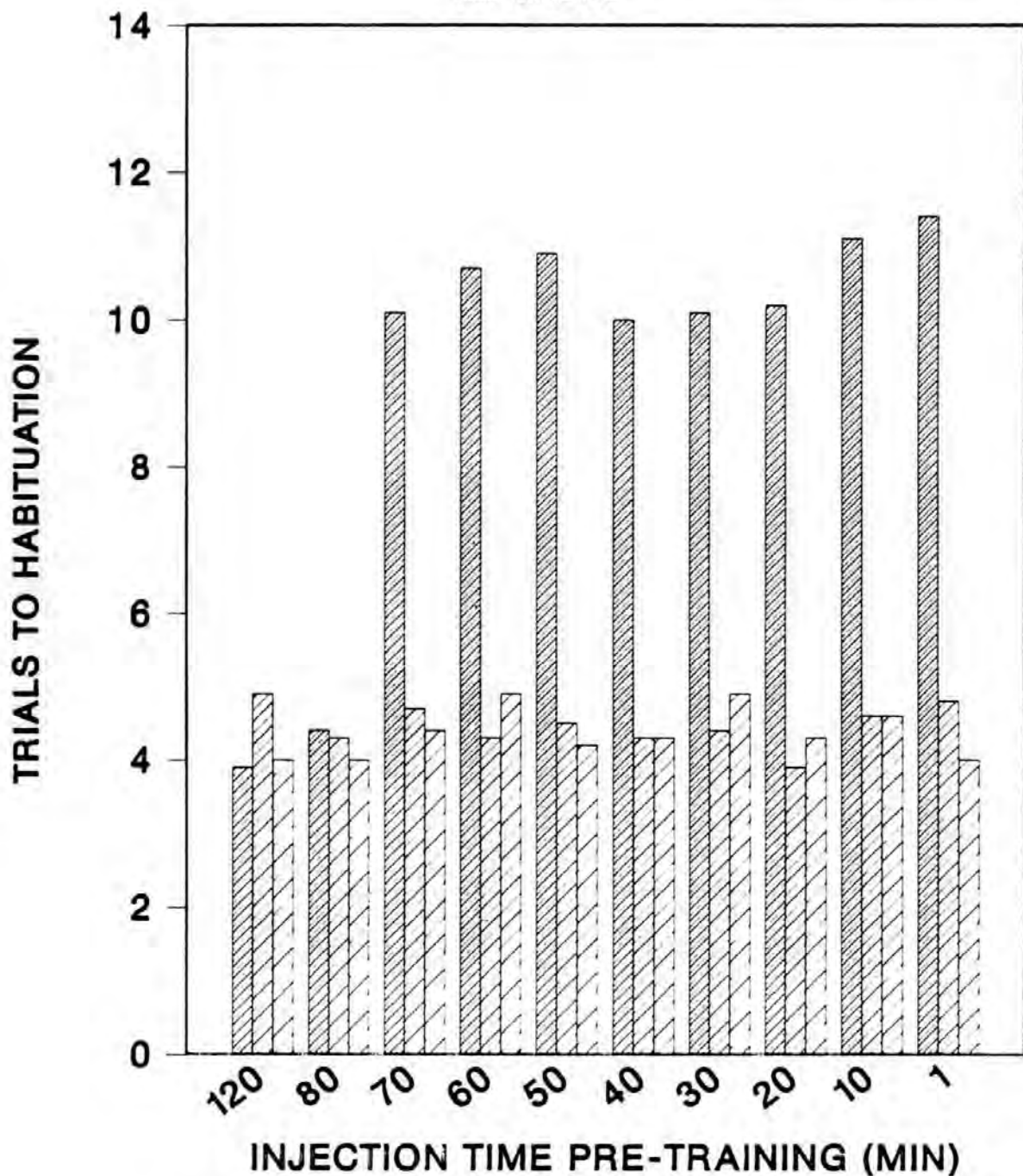
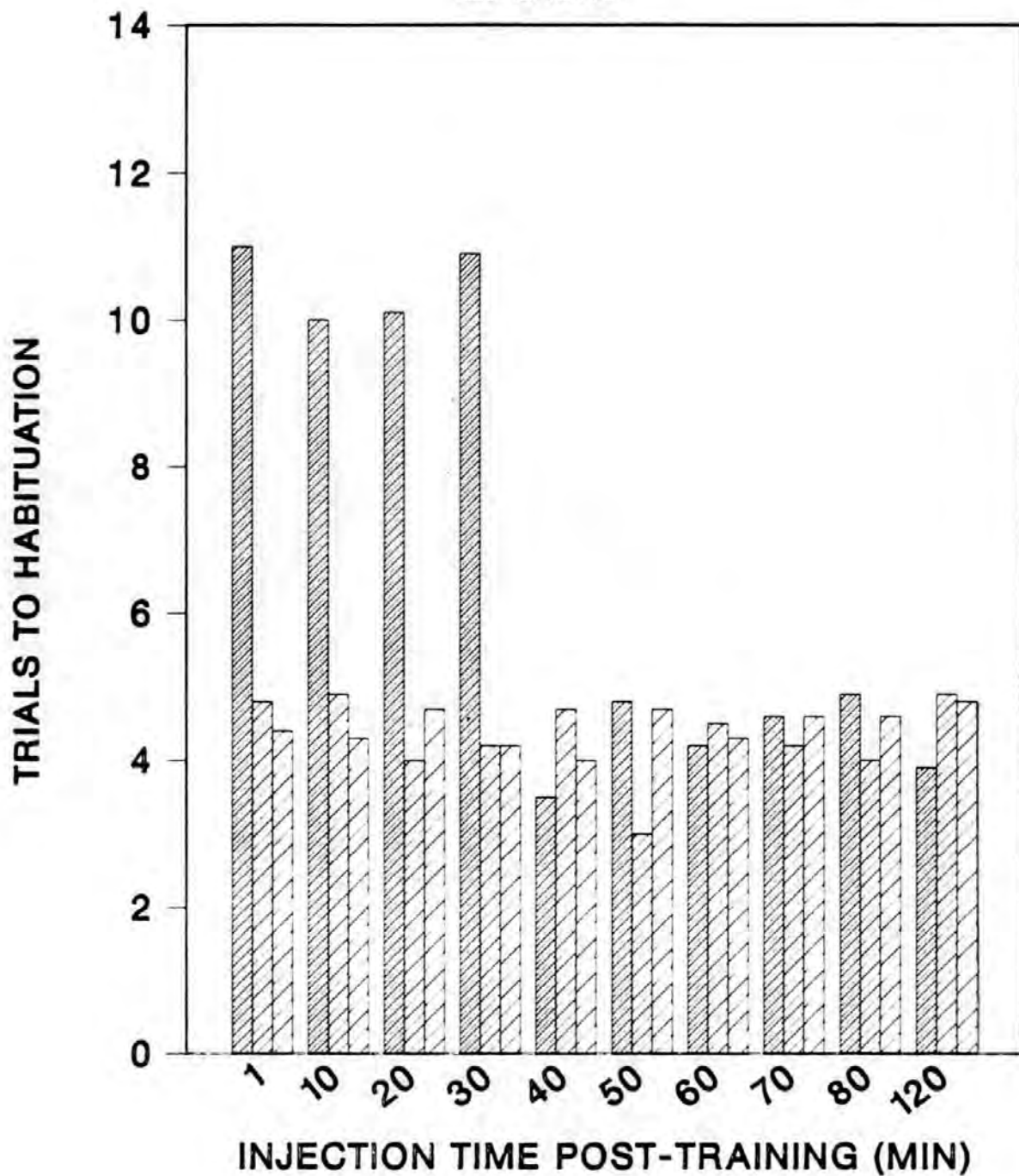


FIGURE 13.24

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 2)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE





DOSE 3 250yg/snail

Analysis of pre training injection times in a 3-way ANOVA revealed significant effects of time ( $F(9/270) = 9.447, p < .001$ ), condition ( $F(2/270) = 256.124, p < .001$ ), and test ( $F(1/270) = 534.287, p < .001$ ) with the following interactions time x condition ( $F(9/270) = 7.913, p < .001$ ), time x test ( $F(9/270) = 5.981, p < .001$ ), condition x test ( $F(1/270) = 62.234, p < .001$ ), and time x condition x test ( $F(9/270) = 5.196, p < .001$ ).

Analysis of simple main effects revealed that short term habituation was not disrupted by the drug injected at any of the pre-training intervals ( $F's(9/270) < 1$ ) (see Figure 13.25). Long term habituation was disrupted by injections of the drug 80 mins pre-training and all lower pre-training injection times ( $F(9/270) < 1$ ). Injections 120 min prior to training had no effect on long term habituation. Snails in this group showed savings at retest compared to training ( $F(9/270) = 572.61, p < .001$ ) (see Figure 13.26).

Analysis of post training injection times at this dose revealed significant effects of time ( $F(9/270) = 5.734, p < .001$ ), condition ( $F(2/270) = 436.987, p < .001$ ), and test ( $F(1/270) = 433.692, p < .001$ ) with significant interactions of time x condition ( $F(9/270) = 12.286, p < .001$ ), time x test ( $F(9/270) = 11.755, p < .001$ ), condition x test ( $F(1/270) = 477.262, p < .001$ ), and time x condition x test ( $F(9/270) = 7.556, p < .001$ ).

FIGURE 13.25

EFFECTS OF PRE-TRAINING INJECTION TIME ON SHORT TERM HABITUATION (DOSE 3)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

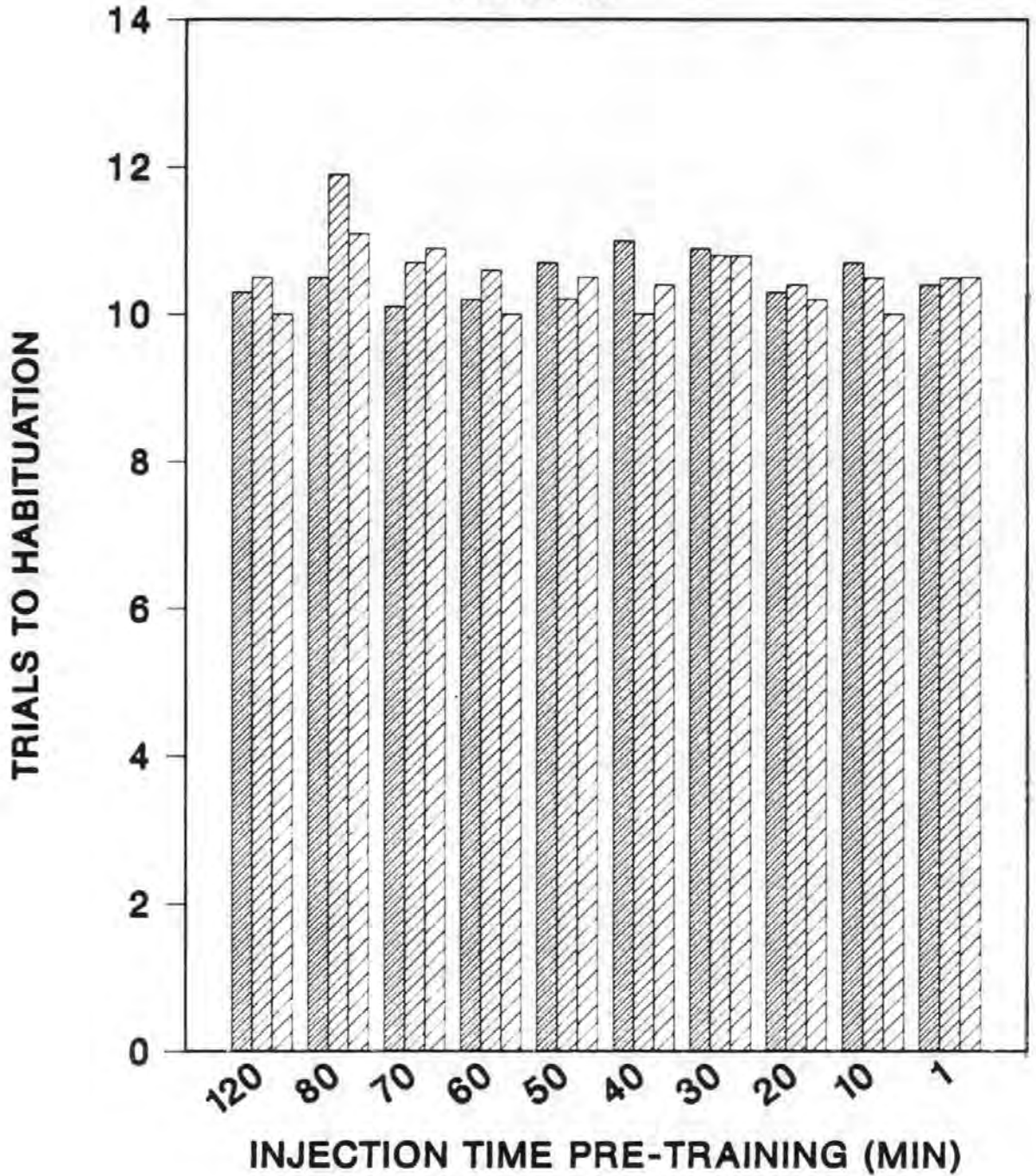


FIGURE 13.26

EFFECTS OF PRE-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE

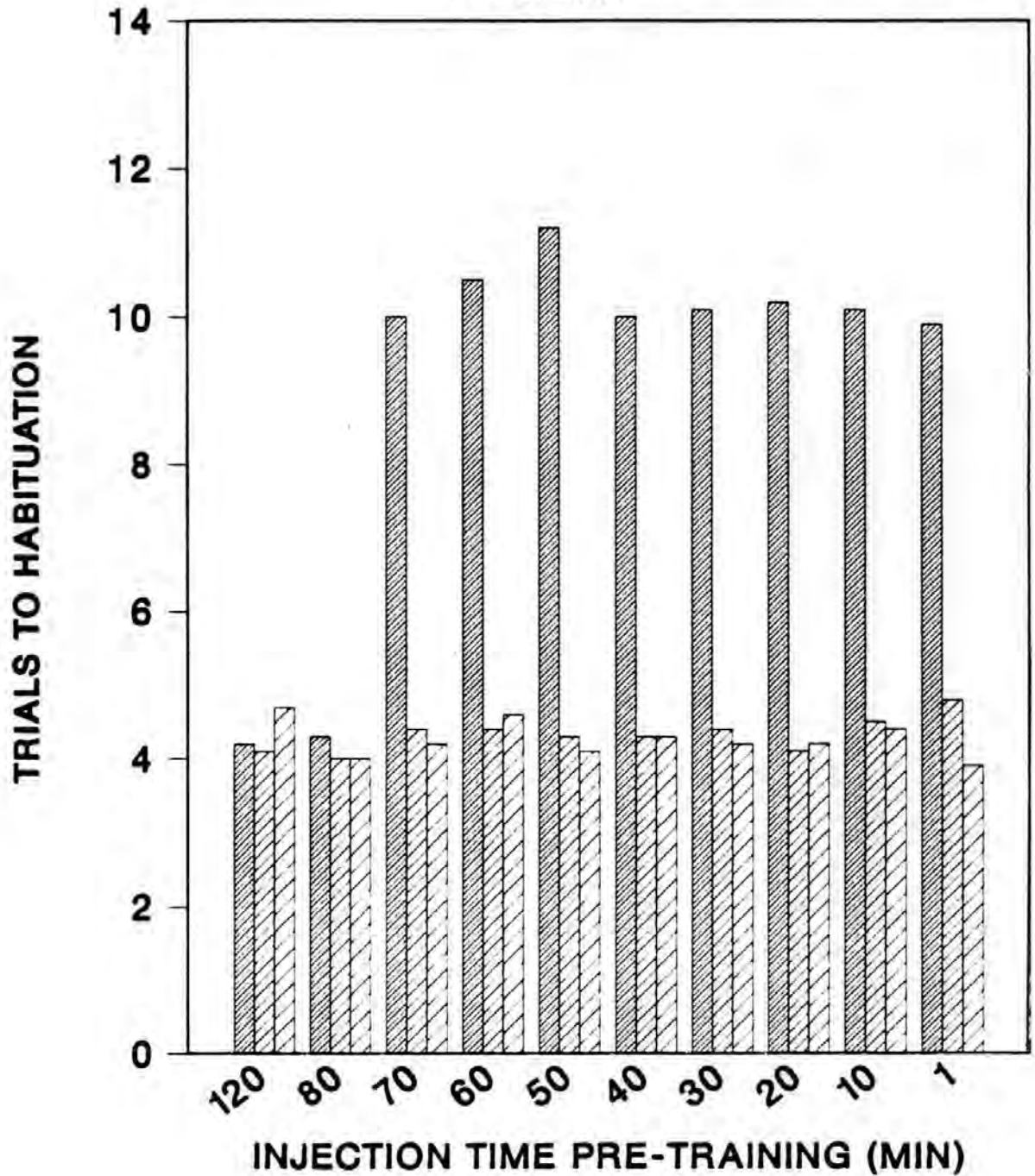
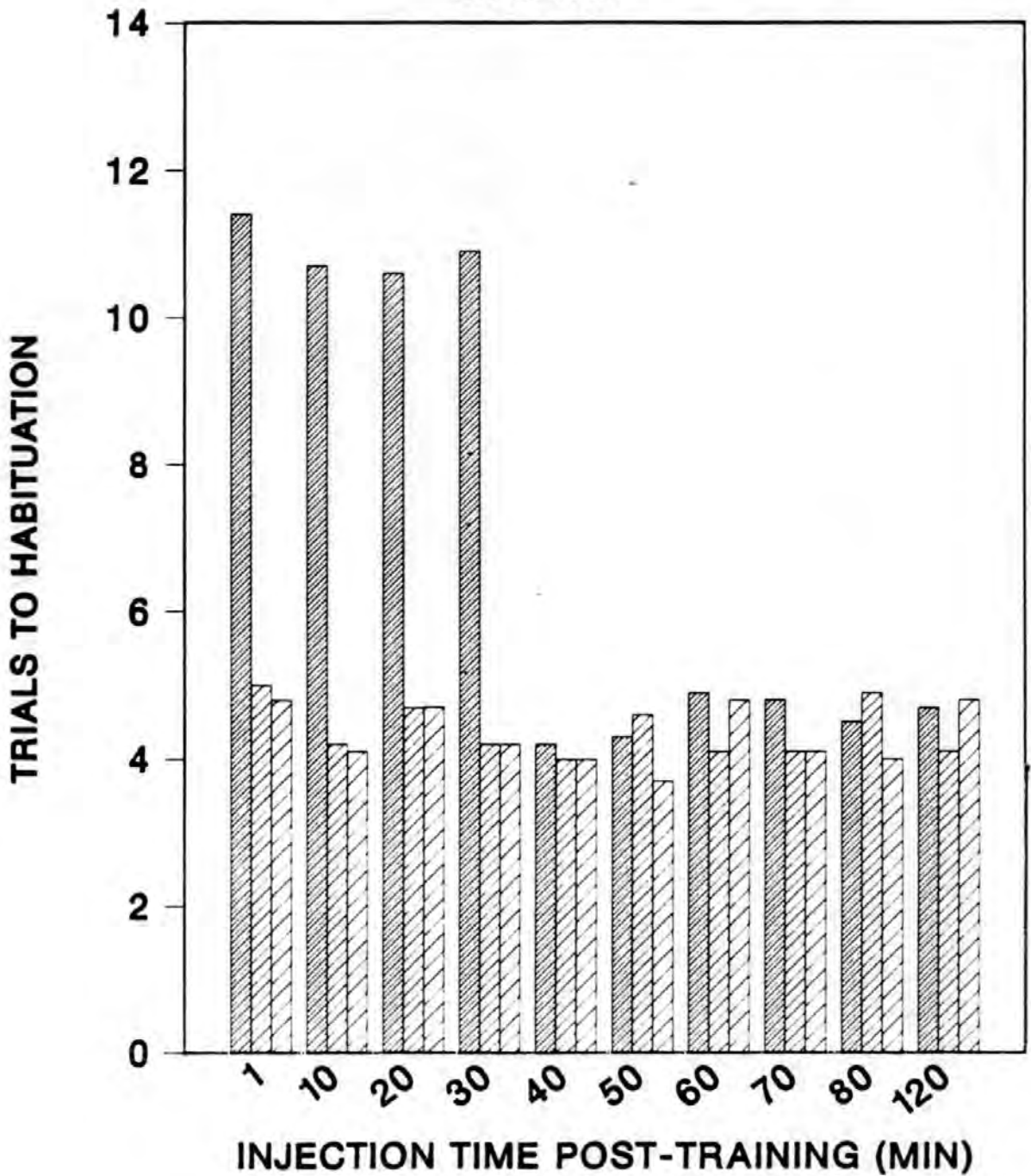


FIGURE 13.27

EFFECTS OF POST-TRAINING INJECTION TIME ON LONG TERM HABITUATION (DOSE 3)

ANISOMYCIN

ANISOMYCIN NO INJECTION  
SALINE



Analysis of simple main effects revealed anisomycin injections up to 40 mins post-training prevented long term habituation ( $F's(9/270) < 1$ ), and from injections times after that, the injections of anisomycin had no effect, and snails in these groups all showed a saving in trials to habituation in retest ( $F's(9/270) = 502.769, 589.548, 617.22, 593.86,$  and  $545.29, p < .001$  respectively). (see Figure 13.27).

## DISCUSSION

From the above experiment it was concluded that long term habituation is dependent on protein synthesis in training and in a brief time window post training of between 30 and 40 mins. Further, dose of anisomycin affects the time the drug can cause the effect when injected pre-training, but as with the other drugs it did not appear to have any major effect on post-training interval. This suggests a critical period of protein synthesis. By comparing groups, the drug appears to remain active, in terms of its behavioural effect, for between 40 and 80 minutes dependent on dose. The results suggest that at Dose 1 the drug could cause its behavioural effect for up to 40 minutes, and, at Dose 2 and 3, for up to 70 minutes.

## GENERAL DISCUSSION

The three experiments reported in this chapter support the conclusions of Squire and Barondes (1976) concerning time of effect of antibiotics and advance their findings by

reporting data from three antibiotics rather than one.

The results demonstrate a critical time period when protein synthesis is necessary for long term habituation to occur. This critical time appears to be during training and for a period of up to 40 mins post training. The results also show a dose effect on the relationship between time of administration of the drug relative to training and the amnesic action of the antibiotic. With the larger doses of all three antibiotics, amnesia could be seen from earlier injection times relative to initiation of training (80 min), whereas, with puromycin and anisomycin, the lowest dose was only capable of producing amnesia with injections 40 minutes or closer to training. There appeared to be no difference in time of effect between anisomycin and puromycin. However, a greater time window of amnesic effect was found with actinomycin D which is an RNA inhibitor. At the lower dose, this PSI could disrupt long term habituation with injections up to 70 minutes pre-training. It is of particular interest to a critical period hypothesis that there was no effect of dose or drug on post-training injection time. With all doses, long term habituation could not be disrupted after 40 minutes post training.

In light of the results of Chapter 7, which demonstrates that these drugs act virtually immediately after a pedal injection, it could be concluded from the studies in the present chapter that the critical period for protein synthesis is during training and in a consolidation period after training lasting between 30 and 40 minutes for this learning in the snail. Results from injections at 120

minutes with all drugs at all doses shows that the drugs are no longer active during training and suggests that they have an observable effect of approximately two hours. It is interesting that these drugs do not appear to have a gradual disruptive effect but are capable of total disruption of long term retention of training within a given time period. The speed of onset of effect is supported by Chapter 7, where the three antibiotics were seen to act immediately, and have a disruptive effect on protein synthesis for between two and two and a half hours. Thus according to the results of this chapter and those of Chapter 7, snails in the 120 min pre-training injection conditions would not have PSI active to a sufficient degree to disrupt long term habituation.

Interestingly, snails in the 120 minute condition pre-training showed normal long term habituation; this provides valuable evidence bearing on the issue of constitutive or specific protein depletion. Early injections would have disrupted pre-existing protein synthesis yet still could not produce amnesia. This suggests that the amnesic effects of the drugs when active in critical period are caused by inhibition of newly expressed protein synthesis necessary for long term learning, and it implies that new gene expression is required for long term habituation in the snail Helix aspersa. A similar conclusion has also been reached by Kandel and his coworkers on *Aplysia* (e.g. Goelet et al., 1986) and by work on single gene mutants in *Drosophila* (Dudai & Quinn, 1980).

It is also of interest that no pre-training injections

of antibiotics had any effect on short term habituation of the response. This supports the findings of experiments reported in Chapters 9, 10, and 11, and research reviewed from other animals with different learning paradigms which was reviewed in Chapter 1.

However, there remains the possibility that there are subtle effects of PSI on short term habituation which measures of progress of habituation do not pick up. This problem is addressed in the next chapter.



## SHORT TERM HABITUATION AND PROTEIN SYNTHESIS INHIBITION

The experiments reported in Chapters 9, 10, 11, 12, and 13 clearly demonstrate the need for protein synthesis in long term habituation and the apparent lack of such a requirement in short term habituation. The conclusion concerning short term habituation is based upon the lack of any effects of PSIs on the progress and speed of short term habituation, and, although this is in agreement with most of the literature (see Chapter 1), there have been suggestions that PSIs do have an effect on short term learning.

Theories of short and long term learning that posit differential mechanisms have relied heavily on PSI evidence. The suggestion that short term habituation is a different biological entity from long term habituation and, further, that short term learning is not dependent on cerebral protein synthesis (e.g. Barondes, 1975; Flood & Jarvick, 1976; Schwartz et al., 1971; Schwartz & Kandel, 1982; Squire, 1975) (see Chapter 1 for review) have been based on research claiming to show that acquisition of learning (short term) is not affected by PSI's. Traditionally, this has been claimed by showing PSI's have no effect on time of acquisition, number of trials to acquisition, or studies designed to look at the time course of short term memory.

While most studies have shown PSI's to have no effect on short term learning, two groups of findings in the literature challenge this basic conclusion. The first is

that it has been reported that PSI's do seem to disrupt short term learning (Gutwein et al., 1974; Quartermain & McEwen, 1970; Rainbow, Alder, & Flexner, 1976; Randt, Barnett, McEwen, & Quartermain, 1971). The second finding was that PSI's seem to prolong retention from short term learning (Quinton, 1978). Such short term retention effects are researched in the next chapter. In this chapter, the suggestion that short term learning is disrupted is further explored in the withdrawal response of the snail.

The suggestion that short term learning is disrupted by PSIs evolved around reports that subcutaneous injections of cycloheximide in mice given shortly before training in a one-trial, step-through, passive avoidance task impaired retention tested within minutes after training (Gutwein et al., 1974; Quartermain & McEwen, 1970; Rainbow et al., 1976; Randt et al., 1971).

These studies however, suffer from methodological problems. First, results for Gutwein's passive avoidance task were subsequently evaluated directly by comparing the effects on short term retention of intracerebral or subcutaneous injection of cycloheximide or anisomycin (Davis et al., 1976). Subcutaneous injection of cycloheximide before passive avoidance training did impair performance at short training-test intervals, as reported by Gutwein et al. (1974) and others. However, protein synthesis inhibition by subcutaneous injection of anisomycin or by intracerebrally injected cycloheximide or anisomycin did not impair performance at the same short intervals after training. In contrast, long term retention of the passive avoidance task

was impaired by both drugs and by both injection protocols. Consequently, it can be seen that the deficit observed shortly after training was unique to subcutaneous injections of cycloheximide and cannot be attributed to inhibition of protein synthesis per se. Furthermore, subcutaneous injections of cycloheximide have been reported to produce increased locomotor activity (see Chapter 8) which is a side effect not shared by subcutaneous injections of anisomycin or intercerebral injections of either PSI (see Chapter 8). It seems likely that impaired short term retention of passive avoidance in the cycloheximide mice may well have been caused by such an increase in locomotor activity.

This view seems all the more compelling in light of the findings that cycloheximide does not impair short term retention in active avoidance or discrimination learning studies (Barondes & Cohen, 1968a; Cohen & Barondes, 1968; Squires & Barondes, 1974).

Nevertheless, there remains the possibility that PSIs do affect short term learning. As was previously stated, the evidence in this thesis that PSIs have no effect on short term habituation is in terms of a lack of effect on the speed and development of short term habituation. It may be that PSIs have a more subtle effect on short term habituation, and that this might be detected by looking for some change in the parametric characteristics of short term habituation.

## EXPERIMENT

### PROTEIN SYNTHESIS INHIBITION AND INTERSTIMULUS INTERVAL EFFECTS ON SHORT TERM HABITUATION

One of the most thoroughly investigated parameters of short term habituation is the effect of ISI, and it is, perhaps, one that has produced most agreement. Experiments in Chapter 5 revealed the expected effect. That is, short ISI's produced faster habituation than long ISI's using a variety of measures of habituation.

Given the robust nature of these findings, the ISI effect seems to provide an ideal vehicle for investigation of any more subtle effect of PSIs which might be occurring.

#### METHOD

Subjects. Two hundred mature Helix aspersa of standard weight (8g) from a population of 300 snails collected from the St. Judes area of Plymouth in wet conditions. All snails were laboratory housed as detailed in Experiment 1, Chapter 3.

Procedure. Snails were randomly allocated to one of 5 ISI conditions: 10 sec, 20 sec, 30 sec, 60 sec, and 300 sec. Forty animals were tested in each ISI condition. Of these, 10 received a 0.1ml injection of physiological saline, 10 received the same volume injections of anisomycin (25yg/snail), 10 puromycin (25yg/snail), and 10 actinomycin D (25yg/snail).

Habituation procedure, stimulus, apparatus orientation period, and criterion of habituation was as reported in Experiment 1, Chapter 5. All data collection was conducted between 0230 and 0800 on the mornings of August 1, 2, 3, 4, 5, 8, 9, 12, 13, and 17, 1986. Temperature in the laboratory was constant at 19 C.

Trials to habituation criterion were recorded for each snail in training and scores compared between pharmacological treatment in different ISIs made.

## RESULTS

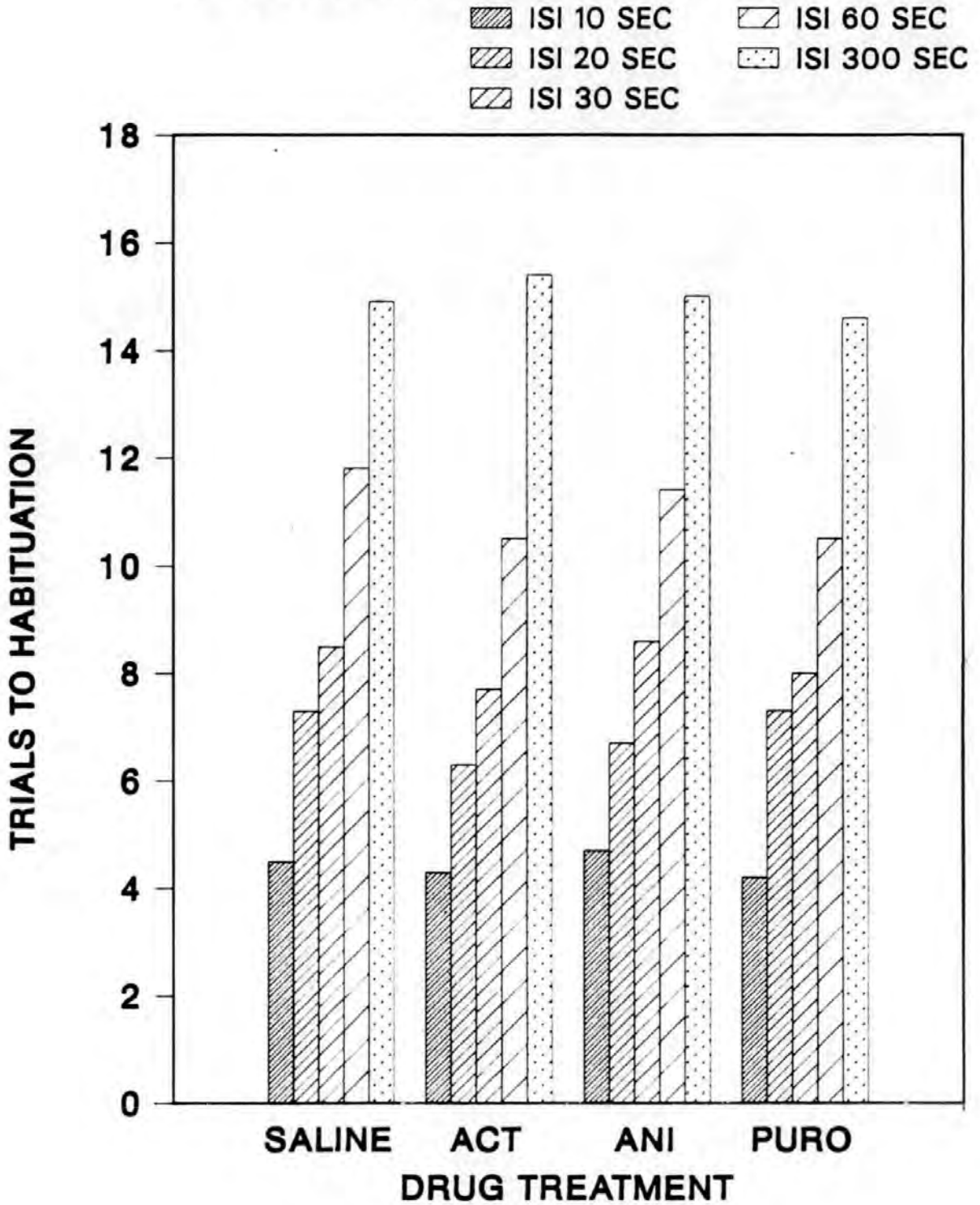
Short term habituation trials to habituation data were analysed in a 2-way ANOVA with two between subject factors of ISI and pharmacological treatment. This revealed a significant effect of ISI ( $F(4/180) = 131.006, p < .001$ ). There was no effect of pharmacological treatment ( $F(3/180) < 1$ ) and no significant interaction ( $F(12/180) < 1$ ). In all pharmacological treatments, a greater number of trials to criterion was required in short term habituation with larger ISI (see Figure 14.1).

## DISCUSSION

Protein synthesis inhibition by three antibiotics did not affect the ISI effect in short term habituation. The results of this experiment provide further evidence in support of other research reported in this thesis that PSI's do not affect short term habituation. ISI effects have

**FIGURE 14.1**

**A COMPARISON OF ISI EFFECTS ON SHORT TERM HABITUATION DURING EPISODES OF PROTEIN SYNTHESIS INHIBITION**



often been cited as a method of studying the mechanisms of short and long term habituation, and their value in such investigations is well established. Further, it has been suggested that short ISIs produce habituation with fewer trials because there is less recovery of response between stimuli (e.g. Carew et al., 1972; Kandel, 1978; Wagner, 1978; 1979, 1981; Whitlow & Wagner, 1981). Evidence presented in this chapter suggests that the recovery of response between stimuli is not affected by protein synthesis inhibition. Thus it was concluded that retention between stimulus presentations was protein synthesis independent.

However, there does remain a problem of definition. How short is short term habituation? For example, it could be argued that the final trials of training may be influenced by long term effects from the first trial of training, and thus the last few trials of training may become subject to PSI effects. The decrement of response analysis in Chapter 11 would argue strongly against such a hypothesis since there was no difference between saline or PSI treated snails at any response in a fixed number of trials experiment. This problem of short term retention and its duration is addressed in the next chapter.

## CHAPTER 15

### EVIDENCE OF SOME RETENTION FROM SHORT TERM HABITUATION IN THE ABSENCE OF PROTEIN SYNTHESIS

The decline in response across trials in training could be considered as evidence of retention across the ISI. This retention appears to be unaffected by PSIs, but the question remains as to how long this period of short term retention can be before it is susceptible to PSI effect.

Normal retention in the presence of PSI's has been reported over short training - test intervals (typically between 1 and 3 hrs) with left/right discrimination for escape and avoidance (Barondes & Cohen, 1966, 1967b; Oliver et al., 1979), light dark discrimination (Barondes & Cohen, 1968a; Cohen & Barondes, 1968b), passive avoidance learning (Davis et al., 1976; Watts & Mark, 1971a, 1971b), and appetitive discrimination learning (Cohen & Barondes, 1968a). Testing of separate groups for retention at longer training - test intervals in the same studies, however, showed retention that was impaired.

While some authors have suggested normal short term retention with PSI's, others have reported that PSI's prolong short term retention. This suggestion was based on the finding that a low, nonamnesic dose of cycloheximide given immediately after training renders retention susceptible to disruption by a second injection of cycloheximide for an unusually long time after training (Quinton, 1978). The hypothesis was that the initial



injection of cycloheximide affected brain metabolism at a time post training that could retard long term memory formation and also prolong short term memory. Consequently, it was suggested that a second dose of the drug could exert an additive effect on weakly formed long term memory and cause amnesia.

Evidence in Helix suggests that multiple post-training injections have no more prolonged amnesic effects than single (see Chapters 9 and 11). Furthermore, the logic of Quinton's argument seems questionable. A more straight forward account would be that the first, non-amnesic dose may simply result in the development of a weakly formed long term learning and lead to an extension of the protein synthesis dependent phase of long term retention. The question as to whether the short term, protein synthesis independent phase of learning is affected or unaffected by PSI's can be addressed in a more direct manner by measuring how long learning persists after training. Davis, Rosenzweig, Jones, & Bennett (1981) carried out such a test and concluded that PSI's do not increase short term learning or its retention. Multiple injections had no effect on recovery compared to single injection treated animals. This result appears to contradict the hypothesis that short term retention can be extended by inhibition of protein synthesis and, instead, supports the conclusion that short term learning is independent of protein synthesis, a conclusion that is in accord with the results in Helix reported in this thesis. However, Davis et al. (1981) and Quinton (1978) remain the only studies with empirical evidence concerning

PSI effects on retention from short term learning.

In order to investigate the duration of short term habituation in *Helix*, two experiments were conducted on the effects of three PSI's on short term habituation of the antennae withdrawal response. An initial study investigated the effects of PSI's on recovery of the response over short post-training periods, and the second investigated retention from short term habituation sampled at hourly intervals post-training.

## EXPERIMENT 1

### PROTEIN SYNTHESIS INHIBITION AND RECOVERY OF RESPONSE FROM SHORT TERM HABITUATION

This experiment investigated recovery of the response after habituation. Experiments reported in Chapter 4 demonstrated that, after criterion of habituation has been achieved, there is some response recovery in terms of initial response at retest. At some point after training the short term process, unaffected by PSIs should decline leaving only long term effects which are susceptible to PSIs. The point at which this occurs is investigated here.

#### METHOD

Subjects. Two hundred snails of standard weight (8g) were used from a population of snails collected from Whitsand Bay, Cornwall. Snails were laboratory housed as

reported in Experiment 1, Chapter 3.

Procedure. Habituation procedure, stimulus, ISI, and criterion of habituation were as reported in Experiment 1, Chapter 4. Twenty snails were tested for recovery at one of 10 post-training intervals, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 min. Of the twenty snails, 5 received saline, 5 anisomycin, 5 actinomycin D, and 5 puromycin (25yg/snail) from a 0.1ml pedal injection 30 min prior to training. The same blind injection procedure as used in all drug studies was applied (see Chapter 10). Recovery was measured by comparing initial retest response magnitude and duration with the initial response in training. Magnitude and duration of response was measured as reported in Chapter 3. Post-training retest time was measured from last no response to initial retest stimulus presentation. Snails remained on the test apparatus in this interval. The experiment was conducted on the mornings of September 2, 3, 5, 6, 7, 8, 9, 11 and 12, 1986 between 0020 and 0740. Temperature in the laboratory was 18 C.

## RESULTS

Recovery of response in each treatment group is illustrated in Fig. 15.1 (saline), Fig. 15.2 (actinomycin D), Fig. 15.3 (anisomycin), and Fig. 15.4 (puromycin). Recovery in terms of initial response durations at training and retest were analysed in a 3-way ANOVA with a within subject factor of test (training/retest) and between subject factors of test time (retest time after training) and

FIGURE 15.1

RECOVERY OF INITIAL RESPONSE AT RETESTS  
IN SALINE INJECTED SNAILS

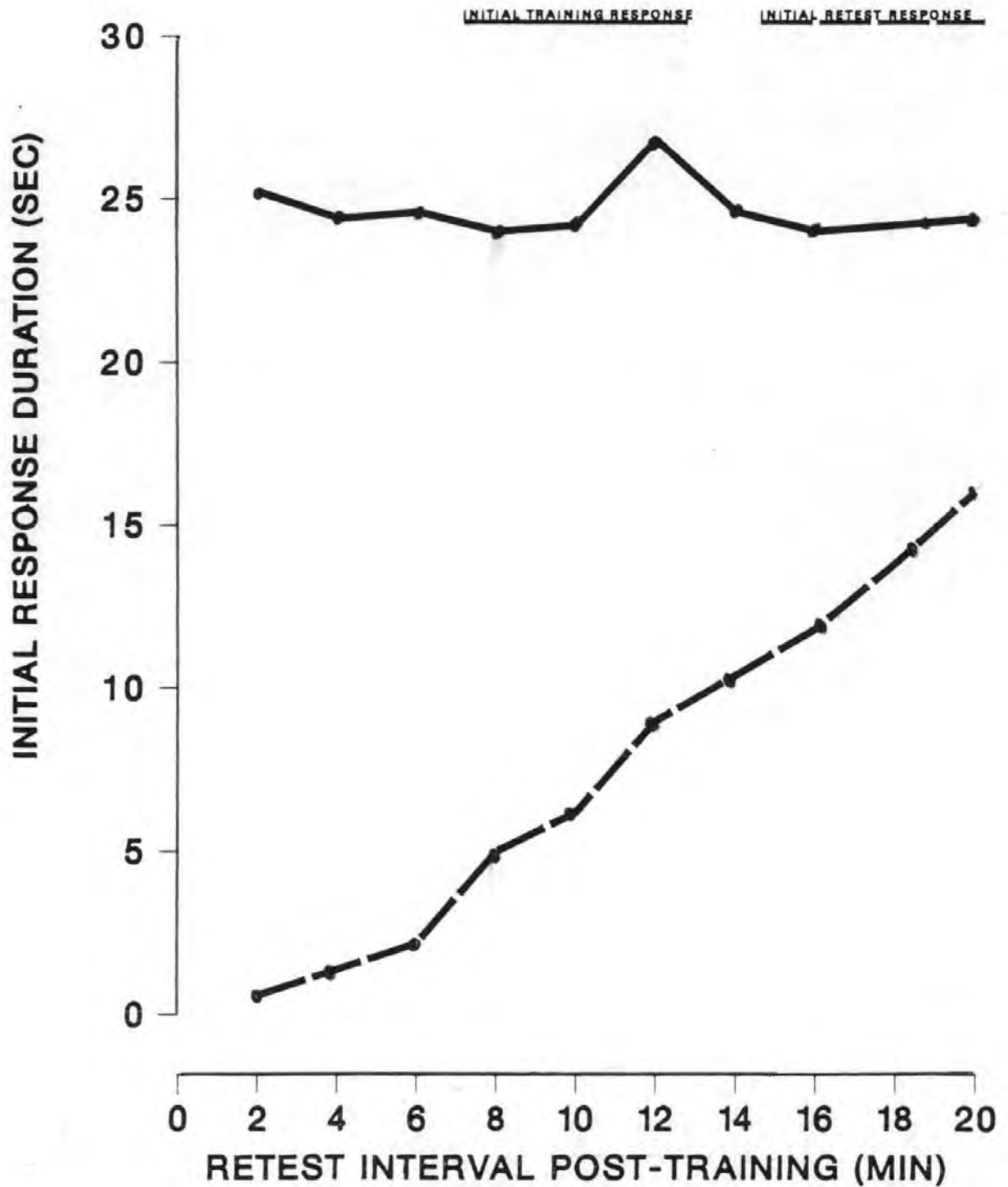


FIGURE 15.2

RECOVERY OF INITIAL RESPONSE AT RETESTS  
IN ACTINOMYCIN D INJECTED SNAILS

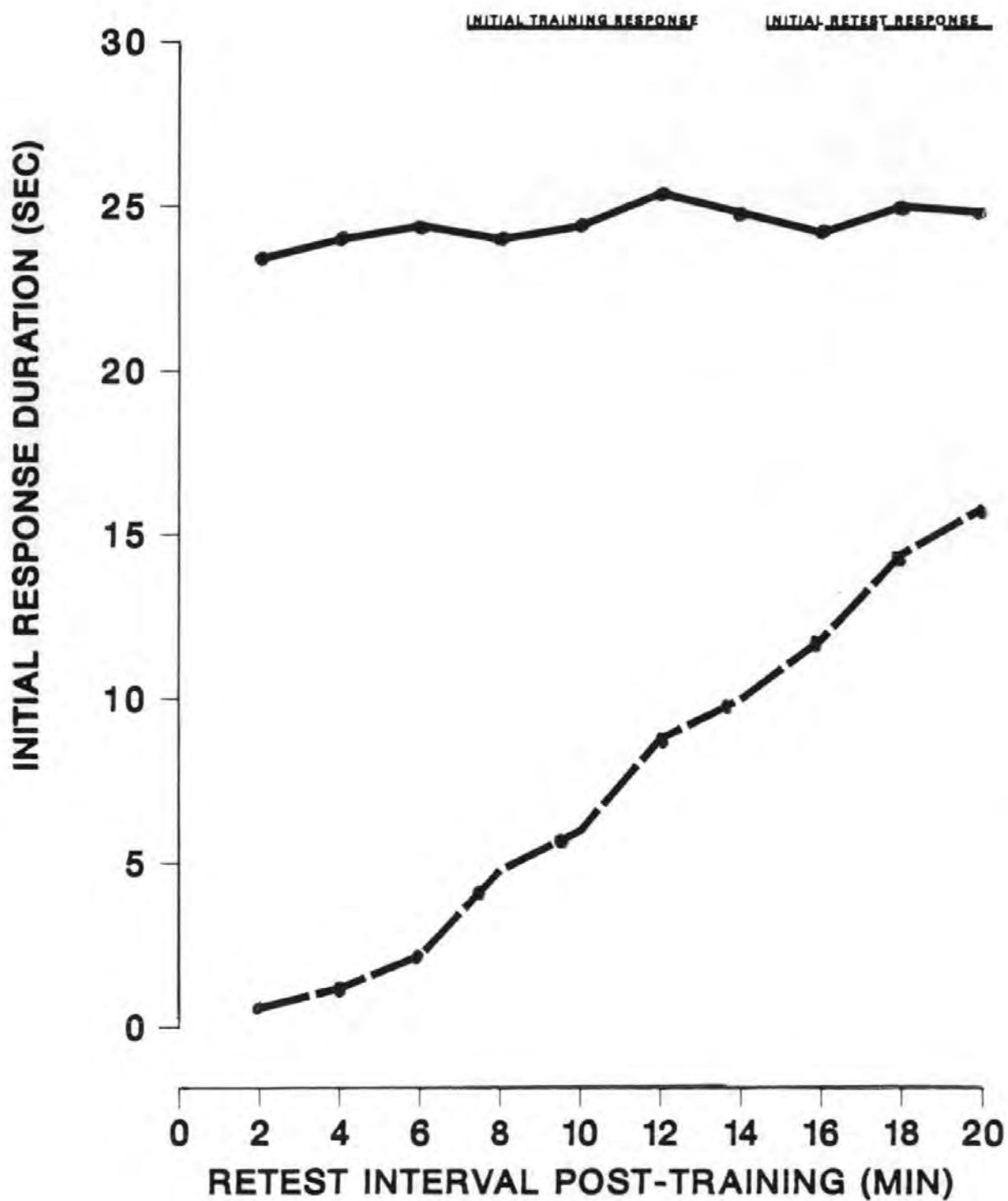


FIGURE 15.3

RECOVERY OF INITIAL RESPONSE AT RETESTS  
IN ANISOMYCIN INJECTED SNAILS

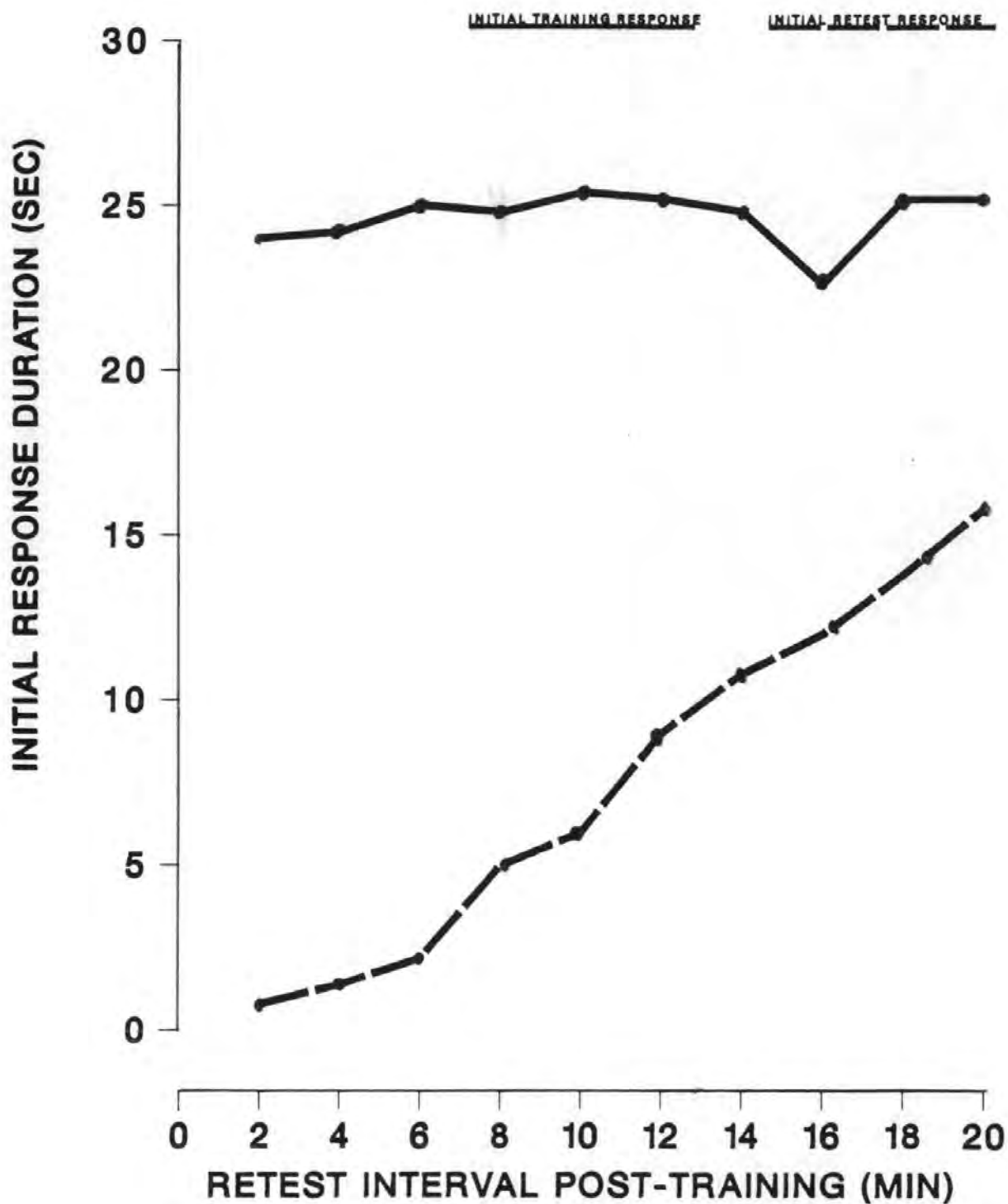
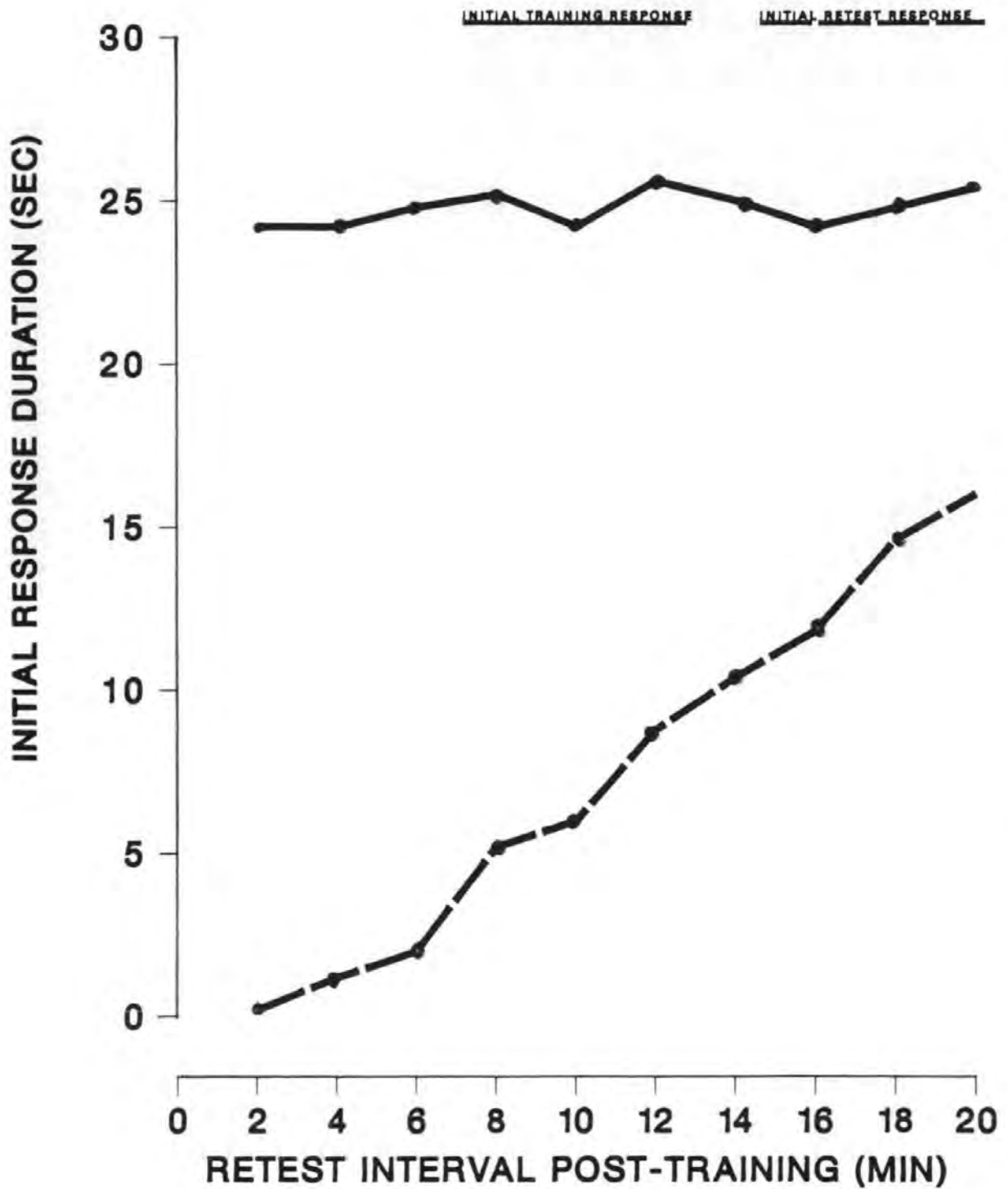


FIGURE 15.4

RECOVERY OF INITIAL RESPONSE AT RETESTS  
IN PUROMYCIN INJECTED SNAILS



treatment. This revealed, a significant effect of test time ( $F(9/160) = 28.996, p < .001$ ) and test ( $F(1/160) = 3703.543, p < .001$ ) with a significant test time x test interaction ( $F(9/160) = 36.281, p < .001$ ). There was no difference between any of the treatments ( $F(3/160) < 1$ ). All other interactions failed to reach significance (all  $F$ 's  $< 1$ ).

Analysis of simple main effects revealed that the response duration at retest had not completely recovered to initial training response level 20 minutes post training ( $F(9/160) = 105.02, p < .001$ ) (see Figure 15.1). There was no difference in response durations between test time groups in the first, initial training trial ( $F(9/160) < 1$ ), but there was a significant difference in retest trial ( $F(9/160) = 1631.93, p < .001$ ) over time. The first response duration in retest increased as interval from training increased showing gradual recovery of response.

Responses were further analysed in terms of their magnitude in a 3-way ANOVA and showed a significant effect of test time ( $F(9/160) = 60.941, p < .001$ ) and test ( $F(1/160) = 136.742, p < .001$ ) with a significant test time x test interaction ( $F(9/160) = 48.561, p < .001$ ). Again, there was no difference between pharmacological treatments ( $F(3/160) = 1.479, p > .5$ ). All other interactions failed to reach significance (all  $F$ 's  $< 1$ ).

Analysis of simple main effects for this measure revealed no significant difference between test time groups in initial training response ( $F(9/160) < 1$ ), but there was a significant difference between first retest responses ( $F(9/160) = 1728.81, p < .001$ ). On this measure response had



recovered in all pharmacological treatments by 8 min post training ( $F(9/160) < 1) p > .5$ ) and recovery was evident at all later retests ( $F's(9/160) < 1$ ).

## DISCUSSION

Utilising two response measures, there was no difference in response recovery post-training between snails that had received antibiotic before training and those in the saline condition. This suggests that the mechanism of short term habituation lasts for at least 20 min in this response. Further, there was no evidence of any of the three PSIs increasing short term retention of habituation as suggested by some authors.

It is also of interest that these data of recovery of response, training, concur with those reported in Chapter 4, Experiment 1.

## EXPERIMENT 2

### PROTEIN SYNTHESIS INHIBITION AND RETENTION FROM SHORT TERM HABITUATION

As the retention durations used in the previous experiment did not appear long enough to capture the point at which protein synthesis independent short term processes give way to the protein synthesis dependent long term processes, a further experiment was carried out with longer retention intervals.

## METHOD

Subjects. One hundred and sixty snails of standard weight collected from a Plymouth garden 1 month prior to experiment and laboratory housed as reported in Experiment 1, Chapter 3 were used.

Procedure. Habituation procedure, stimulus, ISI, and apparatus orientation procedure were as reported in Experiment 1, Chapter 5. All snails were habituated to the tactile stimulus. Forty snails were subsequently tested for retention of learning at each of 4 post-training intervals: 1, 2, 3, and 4 hours. Ten snails received saline, 10 anisomycin, 10 puromycin, and 10 actinomycin D (25yg/snail) from a 0.1 ml pedal injection 30 minutes prior to training. A blind injection procedure was followed (see Chapter 10). Trials to habituation were recorded (exclusive of three non responses) in training and at retest. Retention was evidenced by a saving in retest compared to trial required to habituate in training. A further measure of savings in initial response duration (as used in the previous experiment) was also investigated. All data were collected on the mornings of September 13, 14, 15, 16, 19, 20, and 21, between 2400 and 0930. Temperature in the laboratory varied from 16 to 19 C.

## RESULTS

The results for trials to habituation from Experiment 2

are illustrated in Figures 15.5 (saline), 15.6 (puromycin), 15.7 (actinomycin D), and 15.8 (anisomycin). A 3-way ANOVA, with a within subject factor of test (training/ retest) and between subject factors of treatment (saline, puromycin, actinomycin D and anisomycin) and retest interval (1, 2, 3, and 4 hrs), revealed significant effects of retest interval ( $F(3/144) = 52.262, p < .001$ ), treatment ( $F(3/144) = 47.204, p < .001$ ), and test ( $F(1/144) = 993.300, p < .001$ ) with significant interactions of retest interval x treatment ( $F(3/144) = 6.103, p < .01$ ), retest interval x test ( $F(9/144) = 152.349, p < .001$ ), treatment x test ( $F(3/144) = 176.583, p < .001$ ) and retest interval x treatment x test ( $F(9/144) = 22.195, p < .001$ ).

Analysis of simple main effects revealed that saline injected snails showed significant retention in all retests ( $F's(1/144) = 273.034, 325.936, 348.408, \text{ and } 325.936, p < .001$ ).

With puromycin treated snails, however, retention was only evidenced at the 1 hour retest ( $F(1/144) = 263.015, p < .001$ ). No saving in trials to rehabilitation were found in later retests ( $F's(1/144) < 1$ ). In actinomycin D treatment, again retention was evidenced at 1 hr ( $F(1/144) = 337.078, p < .001$ ), and there was no retention at 2, 3, or 4 hrs ( $F's(1/144) < 1$ ). Similarly, with anisomycin treated snails, retention was only evidenced at 1 hour post-training ( $F(1/144) = 304.213, p < .001$ ), and it was again prevented at later retests ( $F's(1/144) < 1$ ).

The results for initial response duration in training and retest were also analysed in a 3 way ANOVA. This

FIGURE 15.5

RETENTION OF HABITUATION FROM  
SALINE INJECTED SNAILS (CONTROL)

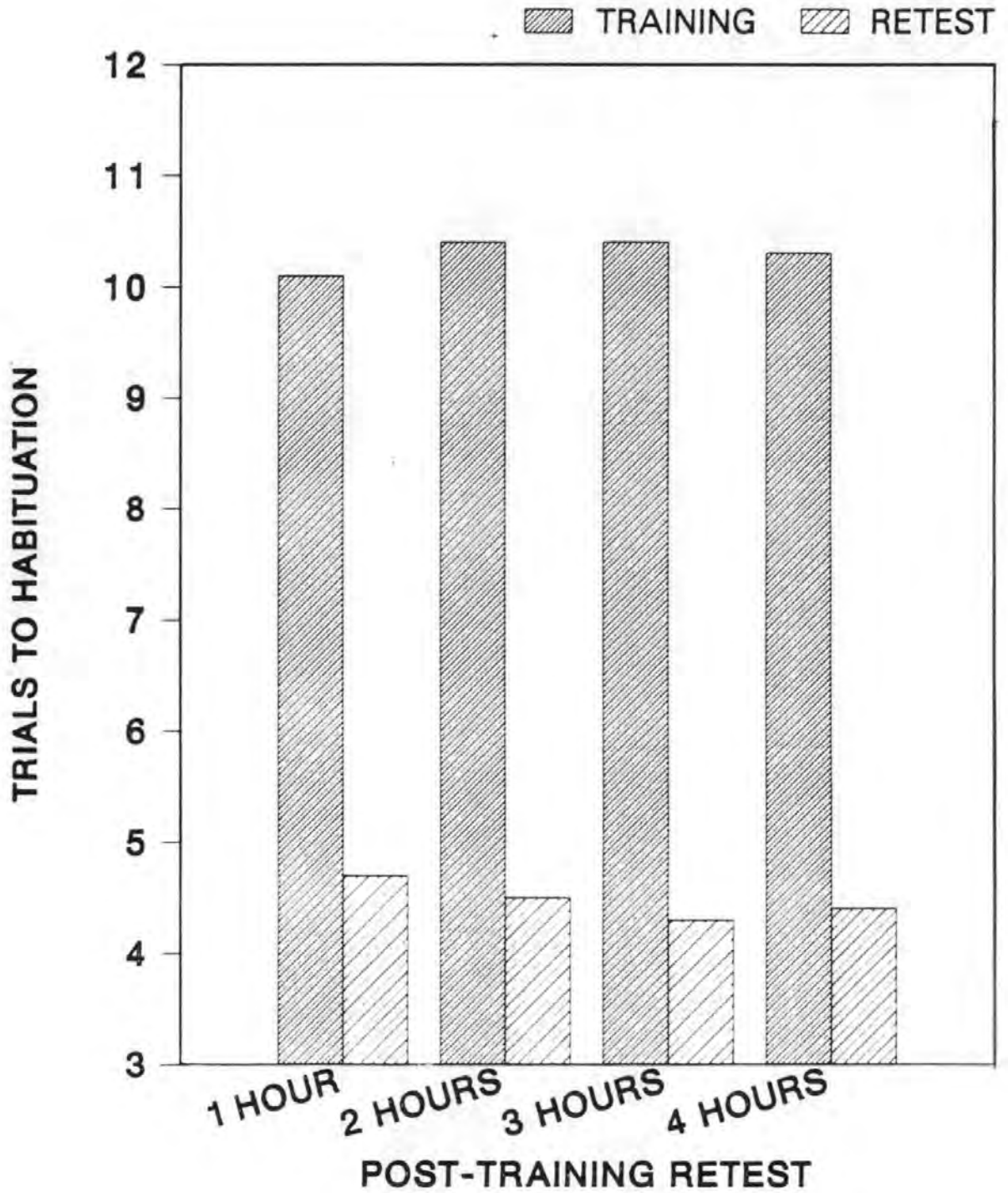


FIGURE 15.6

### RETENTION OF HABITUATION FROM PUROMYCIN INJECTED SNAILS

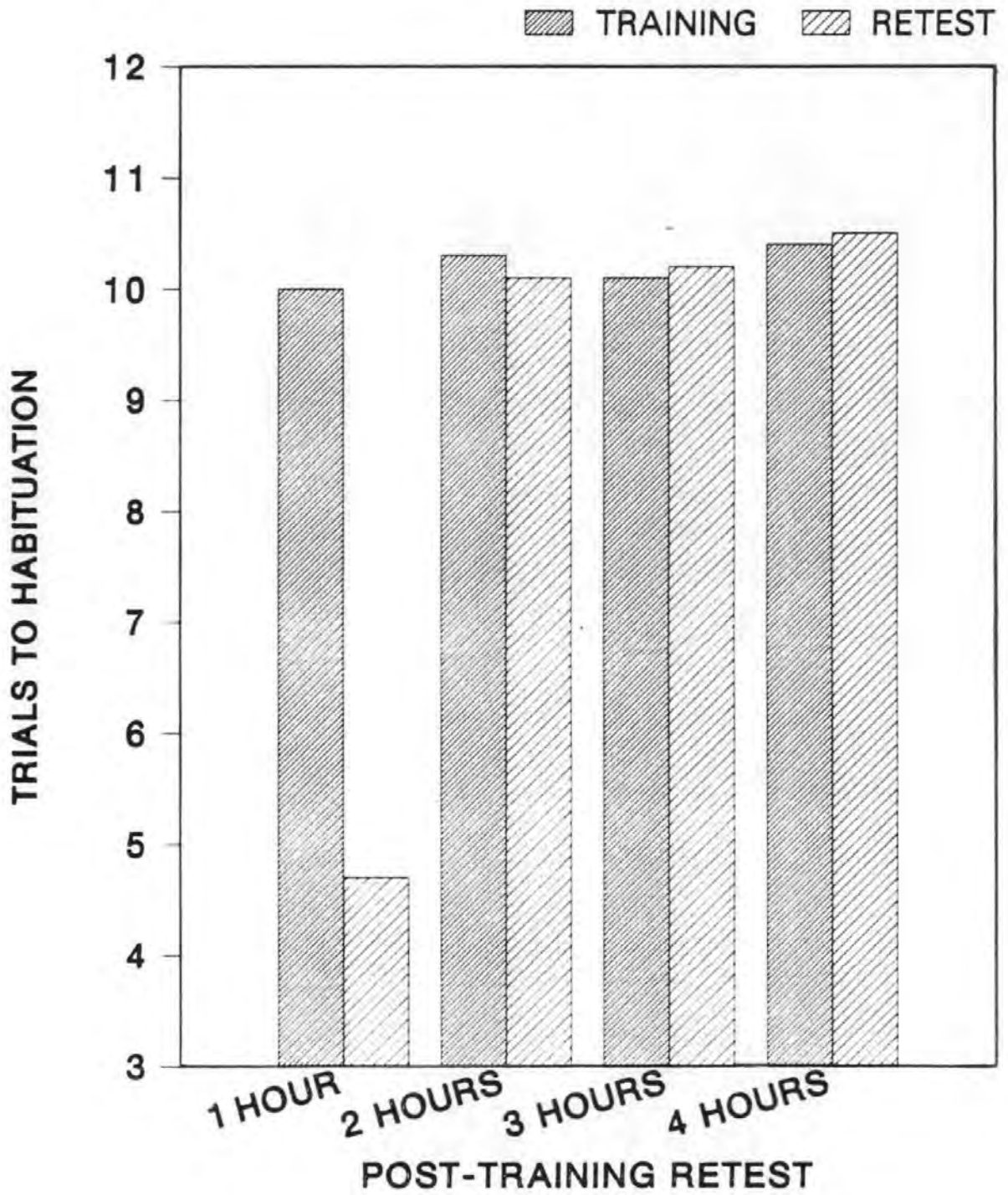


FIGURE 15.7

### RETENTION OF HABITUATION FROM ACTINOMYCIN D INJECTED SNAILS

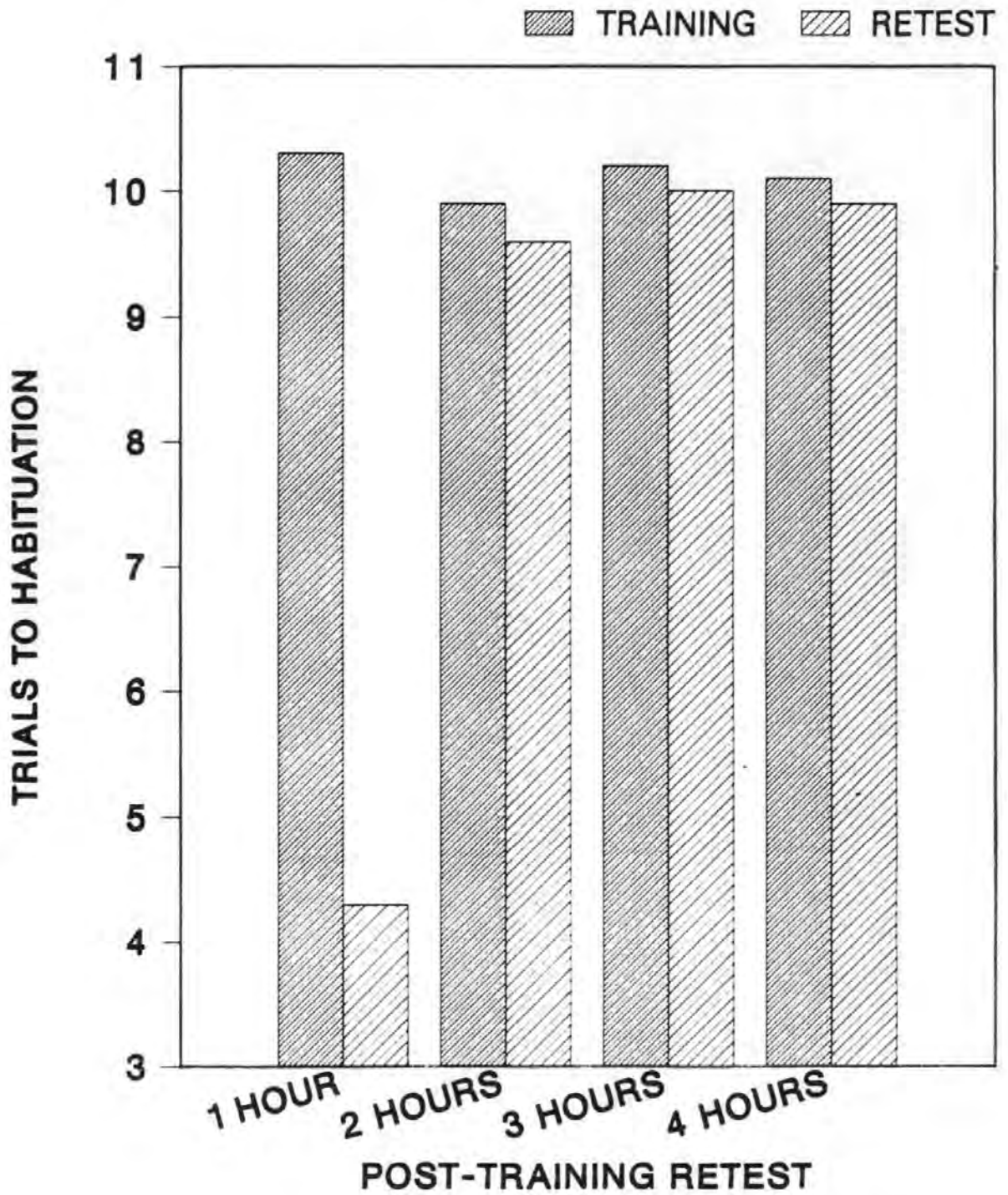
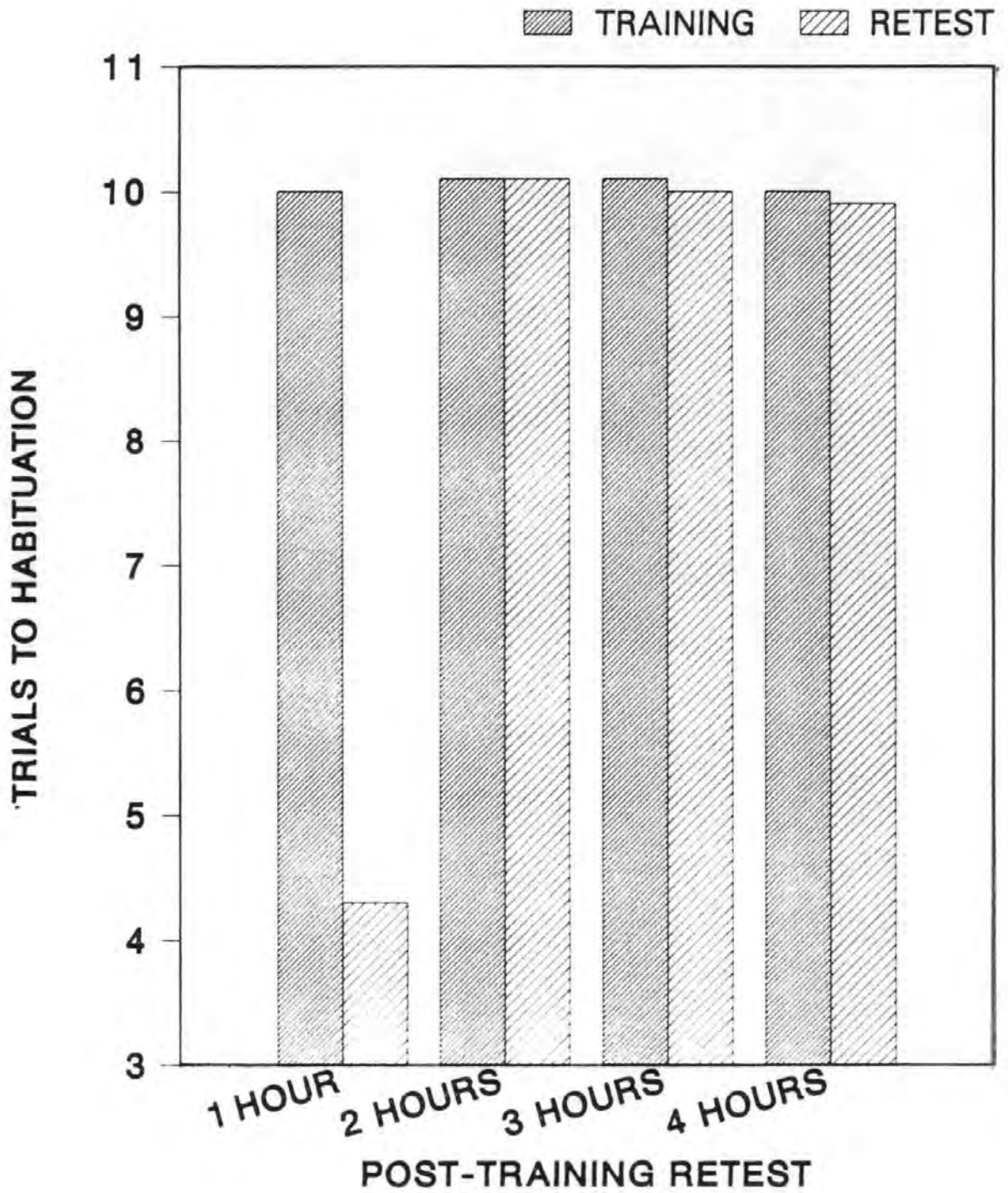


FIGURE 15.8

### RETENTION OF HABITUATION FROM ANISOMYCIN INJECTED SNAILS



revealed significant effects of retest interval ( $F(3/144) = 67.28, p < .001$ ), treatment ( $F(3/144) = 50.94, p < .001$ ) and test ( $F(1/144) = 879.994, p < .001$ ) with significant interactions of retest interval x treatment ( $F(3/144) = 8.021, p < .001$ ), retest interval x test ( $F(9/144) = 162.743, p < .001$ ), treatment x test ( $F(3/144) = 194.328, p < .001$ ), and retest x treatment x test ( $F(9/144) = 20.927, p < .001$ ).

Analysis of simple main effects revealed that saline injected snails showed significant retention in all retests ( $F(1/144) = 199.87, 268.52, 320.46, \text{ and } 247.61, p < .001$  respectively for snails tested 1, 2, 3, or 4 hours post training).

From puromycin treated snails, however, retention was only evidenced at 1 hour post training ( $F(1/144) = 294.852, p < .001$ ). No savings in initial retest response compared to initial training response were found in later retests ( $F(1/144) < 1$ ).

In actinomycin treated snails, again, no retention was found after the 1 hour retest ( $F(1/144) < 1$ ). Snails did show retention on this measure in the 1 hour test ( $F(1/144) = 308.721, p < .001$ ).

The same effects were found in anisomycin treated snails. There was retention at 1 hour post training ( $F(1/144) = 268.996, p < .001$ ) but not at later post training retests ( $F's(1/144) < 1$ ).



## DISCUSSION

Retention was evidence in all retests from saline injected snails. However, in the protein synthesis inhibition treatment groups, there was no retention evidenced beyond 1 hour post-training, whereas retention was evidenced in all PSI groups at 1 hour post training. This result further indicates that the mechanism of short term habituation is protein synthesis independent and that this mechanism does itself support some retention as other research using different learning behaviours had reported (see Davis & Squire, 1984 for review).

### GENERAL DISCUSSION

Taking the results of both experiments, retention of habituation appears unaffected by PSIs up to and including 1 hour post-training, but is affected at 2 hours post training and beyond. The duration of short term retention appears, therefore, to be somewhere between 1 and 2 hrs. Any parallel long term processes which might be affected by protein synthesis inhibition did not have an effect on expressed retention up to 1 hour post-training. It would seem from this that, for this period at least, long term habituation processes play little part in the expression of retention of learning.

The results of the two experiments reported in this chapter appear to conflict with Quinton's suggestion that protein synthesis inhibition can prolong short term

processes. Research in both above experiments demonstrates that there was no difference in recovery of response post-training between saline injected snails and PSI injected snails, and this was true in terms of initial recovery of response in terms of minutes (Experiment 1) and recovery of response over hours (Experiment 2).

The differential effects of PSIs in short and long term habituation explored thus far in the thesis were then further investigated in terms of the degree of amnesia PSIs can induce in a long term habituation test.

## CHAPTER 16

### EXTENT OF AMNESTIC EFFECT IN LONG TERM HABITUATION FOLLOWING PROTEIN SYNTHESIS

The effects of protein synthesis inhibition has traditionally established long term effects by a comparison of performance in training and at retest (see Chapter 1). While this approach is adequate to demonstrate that protein synthesis inhibition has an effect on long term learning, it does not serve to show the extent of the amnesia. It may be that there is some retention, and this may manifest itself in terms of the parametric characteristics of long term habituation.

If the effect on long term habituation demonstrated in previous chapters is complete amnesia, the parametric characteristics of the habituation evidenced at retest should reflect those previously demonstrated for short term habituation as opposed to those established for long term habituation. In order to investigate this, the differential effects of ISI on short and long term habituation were employed (see Chapter 5). This demonstrated that ISI has no effect on long term habituation, but, in short term habituation, short ISIs produce faster habituation. Thus, if the snail was made completely amnesic by the PSI in the critical time window established in earlier chapters, the learning demonstrated at retest should reflect the effects of ISI in short term habituation rather than long term.

## GENERAL METHOD

Three experiments were conducted using the following general method to investigate the amnesic properties of anisomycin, actinomycin D, and puromycin.

Subjects. One hundred and twenty snails of standard weight collected from the Stonehouse area of Plymouth were employed in each experiment. Subjects were laboratory housed as reported in Experiment 1, Chapter 3.

Procedure. Forty snails were sampled in each of three ISI conditions, 30 secs, 60 secs, and 300 secs. Within each ISI condition, 20 snails received a pedal injection of the antibiotic and 20 snails a pedal injection of physiological saline. All injections were carried out 20 mins prior to the training session. Of the twenty snails in each treatment condition, 10 Snails received training to criterion of habituation followed by a retest 12 hrs post-training, 10 received no initial training before test. Instead of training these snails were still handled and placed on the laboratory test board. Intervals between sessions were spent in the home vivarium.

Habituation stimulus, test apparatus and apparatus orientation period were as reported in Experiment 1, Chapter 5. The ISI for the snail, dependent on group, was used in both training and retest. In all three experiments dose of drug was 25 yg/snail from a 0.1 ml injection. Saline injections were with an equal volume and the blind injection procedure was followed (see Chapter 10).

## EXPERIMENT 1

### AMNESTIC EFFECTS OF PUROMYCIN IN LONG TERM HABITUATION

The first experiment used the above methodology to investigate the amnesic effect of the antibiotic puromycin. The experiment was conducted between 2100 and 1015 hrs on October 1 to 9, 1986. Temperature in the laboratory varied from 17 to 20 C.

### RESULTS

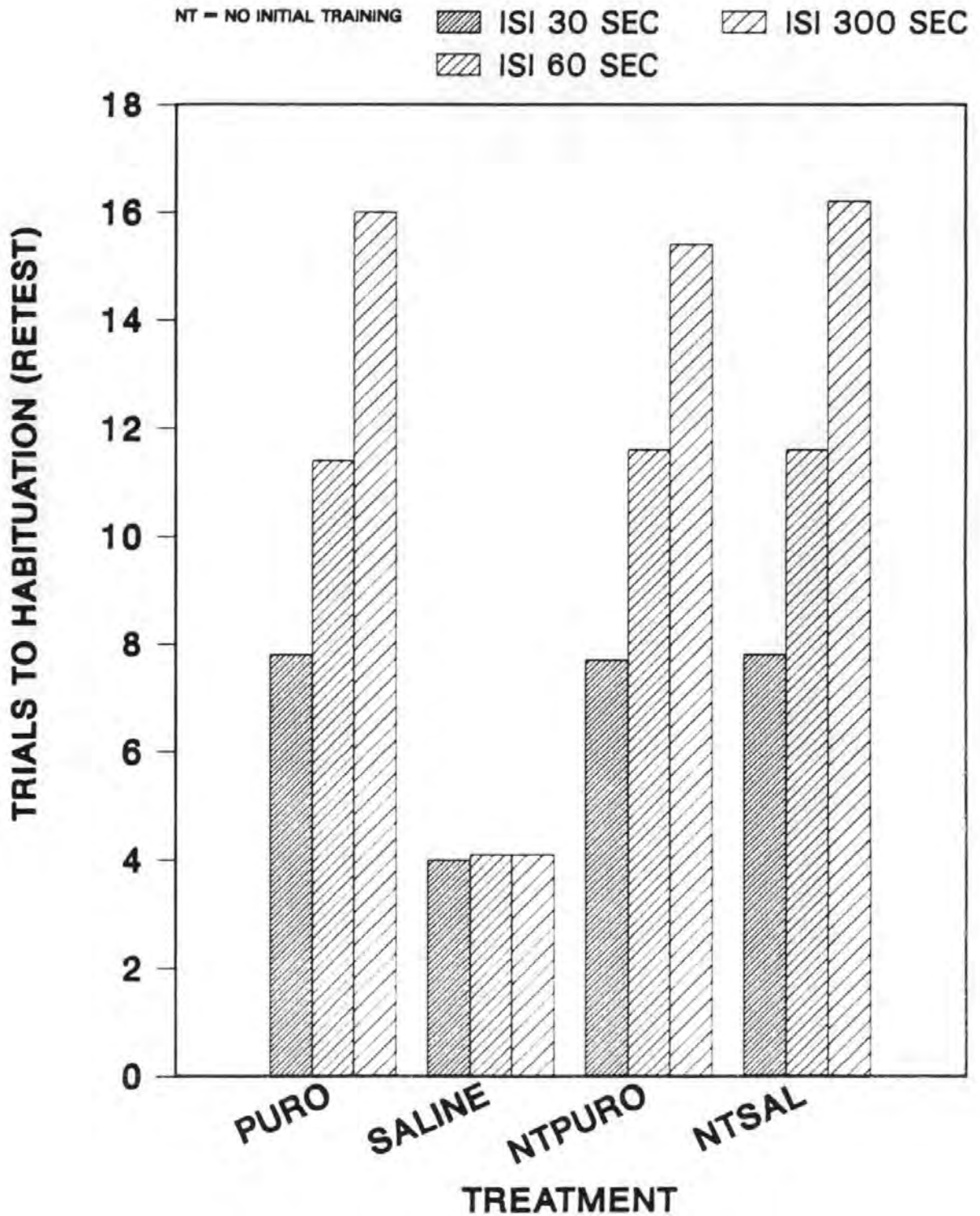
The effects of ISI on puromycin and saline treated snails in a long term habituation retest are illustrated in Figure 16.1. Analysis in a 2-way ANOVA with between subject factors of ISI and condition (drug/saline) revealed significant effects of ISI ( $F(2/108) = 129.695, p < .001$ ) and condition ( $F(3/108) = 153.145, p < .001$ ) with a significant ISI x condition interaction ( $F(6/108) = 14.069, p < .001$ ).

Analysis of simple main effects revealed that saline injected snails showed no effect of ISI on trials to habituation in the long term retest ( $F(6/108) < 1$ ) and required fewer trials to rehabilitate than drug treated pre-trained snails ( $F(6/108) = 429.082, p < .001$ ). Retest scores in the puromycin injected, pre trained snails showed short ISI's produced faster habituation than long ISI's ( $F(6/108) = 109.978, p < .001$ ).

Comparisons between no-training saline and drug groups revealed short ISI conditions required fewer trials to

FIGURE 16.1

THE EFFECTS OF ISI MANIPULATION IN A 12 HOUR RETEST WITH VARIOUS PUROMYCIN TREATMENTS



habituation in both drug and saline groups. There was no difference in these retest scores between treatments ( $F'_{s(6/108)} < 1$ )

## DISCUSSION

Puromycin treated snails showed no long term habituation. Further, there was an ISI effect typical of short term habituation present in the long term habituation test in the puromycin snails that was absent in saline injected animals. Saline injected snails showed no effect of ISI on trials to habituation at retest. Thus, it was concluded that puromycin induced amnesia is total. Drug treated, trained animals showed no difference from snails that had received no training in terms of parametric characteristics of habituation.

## EXPERIMENT 2

### AMNESTIC EFFECT OF ANISOMYCIN IN LONG TERM HABITUATION

The above experiment was replicated with the antibiotic drug anisomycin at a known safe dose (25yg/snail) on the nights of October 10 to 19, 1986 between 1600 and 1030 hrs. Laboratory temperature varied from 19 to 21 C.

## RESULTS

Data for Experiment 2 is presented in Figure 16.2. Analysis in a 2-way ANOVA (see Experiment 1) revealed significant effects of ISI ( $F(2/108) = 128.919, p < .001$ ) and condition ( $F(3/108) = 187.892, p < .001$ ) with a significant interaction of ISI x condition ( $F(6/108) = 14.876, p < .001$ ).

Analysis of simple main effects showed there was no ISI effect in long term habituation in saline injected snails ( $F(6/108) = 0.003, p > .5$ ), but, an ISI effect in long term habituation in anisomycin treated snails ( $F(6/108) = 89.593, p < .001$ ) with short ISIs required fewer trials to criterion than long ISIs.

Comparisons between no-training groups again revealed the superiority of short ISIs in terms of trials to habituation. There was no difference between saline and drug, no training scores for any ISI ( $F's(6/108) < 1$ ).

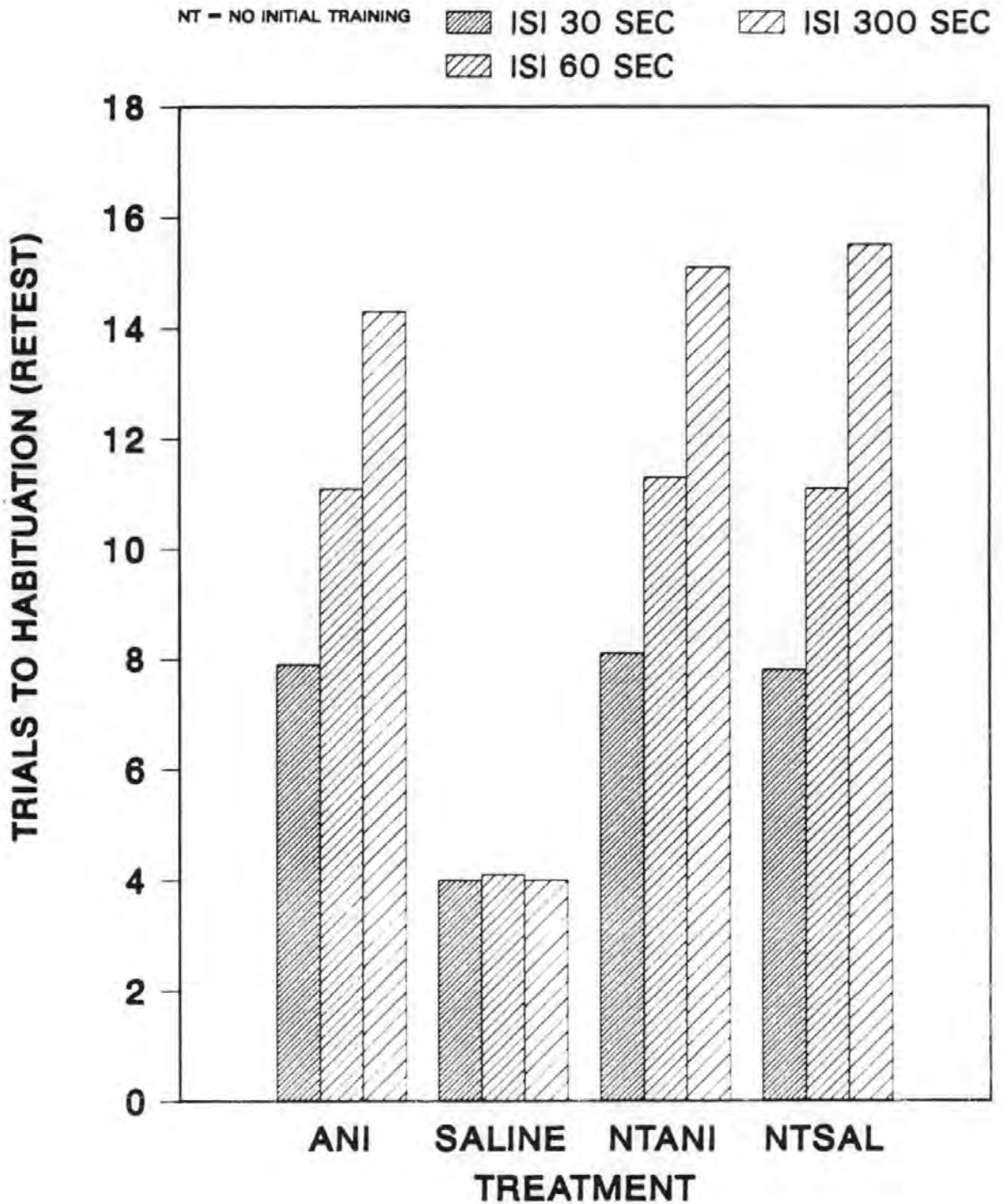
## DISCUSSION

Anisomycin injected prior to training produced amnesia in retest which was total. Further, the parametric characteristics of the learning evidenced in retest by the trained, drug treated snails was consistent with short term habituation rather than the characteristics of long term habituation evidenced by the saline treated trained snails. Thus anisomycin treated snails were totally amnesic with regard to original training.



FIGURE 16.2

THE EFFECTS OF ISI MANIPULATION IN A 12 HOUR RETEST WITH VARIOUS ANISOMYCIN TREATMENTS



### EXPERIMENT 3

#### AMNESTIC EFFECT OF ACTINOMYCIN D ON LONG TERM HABITUATION

A further experiment was conducted with a third antibiotic actinomycin D (25yg/snail). This experiment was conducted October 20 to 30, 1986, between 1600 and 1230. Temperature in the laboratory was a constant 19 C.

#### RESULTS

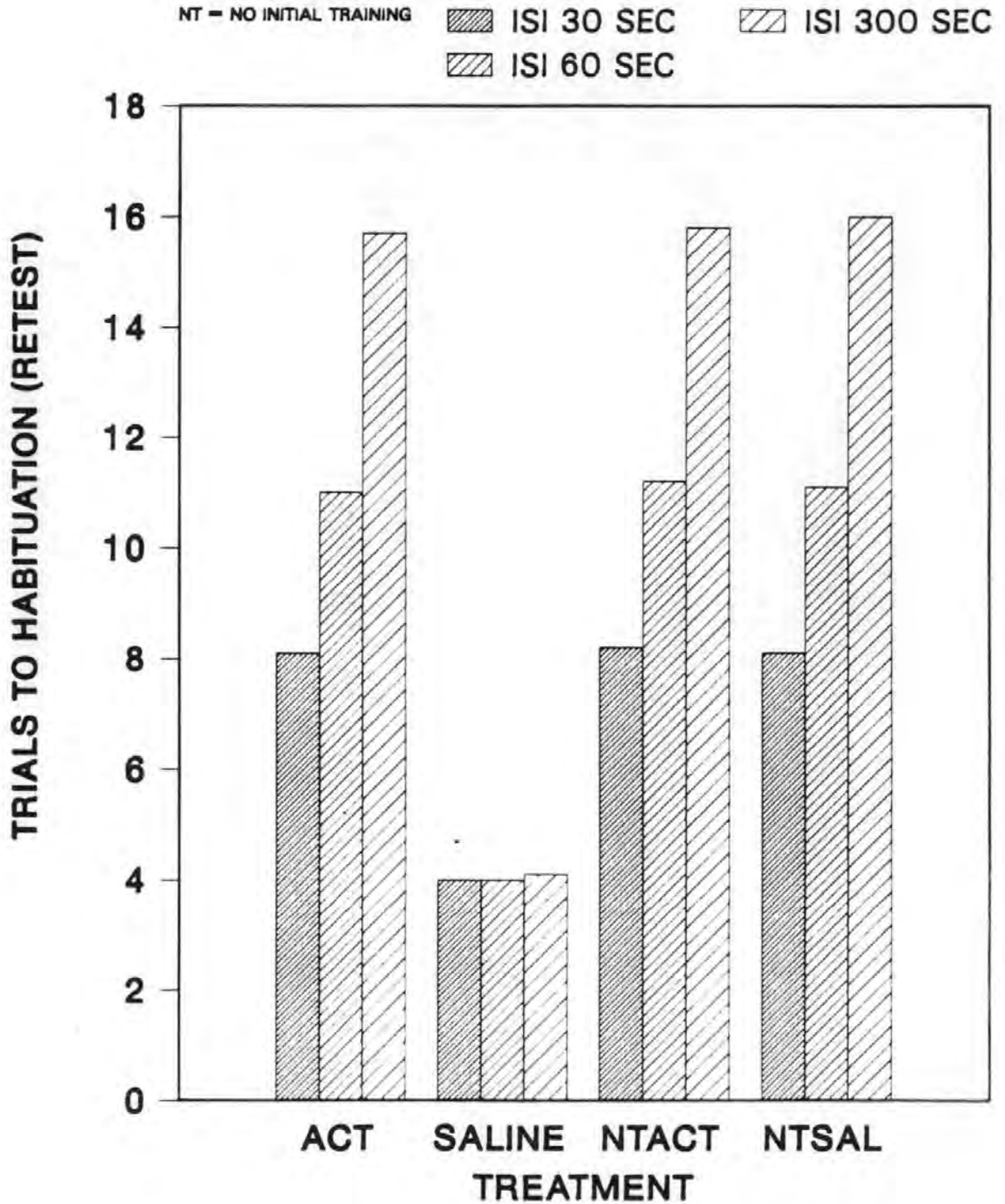
Trials to habituation during retest data are reported in Figure 16.3. Analysis of these data in a 2-way ANOVA shows a significant effect of ISI ( $F(2/108) = 159.085$ ,  $p < .001$ ) and condition ( $F(3/108) = 204.236$ ,  $p < .001$ ) with a significant ISI x condition interaction ( $F(6/108) = 17.067$ ,  $p < .001$ ).

Analysis of simple main effects again revealed saline injected snails to exhibit no effect of ISI on retest ( $F(6/108) < 1$ ). Snails in the drug condition showed an effect of ISI, with short ISI's again requiring fewer trials to criterion than long ISI's.

Analysis of no training groups revealed no difference between the treatments. Both saline and actinomycin D injected no-training snails required fewer trials to reach criterion of habituation with shorter ISIs.

FIGURE 16.3

THE EFFECTS OF ISI MANIPULATION IN A 12 HOUR RETEST WITH VARIOUS ACTINOMYCIN D TREATMENTS



## DISCUSSION

The results show that actinomycin D produced total amnesia of original training with treated snails showing no long term habituation, and the development of habituation in retest showed the parameters equating to a short term habituation process.

### GENERAL DISCUSSION

The above three experiments demonstrated that when PSIs are active in short term habituation, retention sampled after 12 hrs showed the parametric characteristics of short term habituation. Saline injected snails showed no effect of ISI in retest, whereas PSI injected snail showed habituation to criterion with fewer trials in the shorter ISI conditions. This ISI effect was only observed in short term habituation when protein synthesis remained intact. The retest ISI effect in PSI treated snails was identical that demonstrated by snails which had received no prior training. This shows that, in the antennae withdrawal response in the snail Helix aspersa, protein synthesis inhibition in the critical time window completely blocked long term habituation with inhibited animals behaving as though they had no prior experience of the stimulus.

The communality of results between the experiments is particularly significant in that each of the three antibiotics have been demonstrated to disrupt protein synthesis by a different modes of action. This, and other

theoretical implications of these three experiments will be discussed in Chapter 18.

If the animal can remember nothing of the initial training, as these experiments suggest, PSI treated snails should not exhibit an effect of multiple training sessions that is typical of non drug treated animals (see Chapter 4). The next chapter investigates the effects of PSIs on multiple training sessions.

## CHAPTER 17

### PROTEIN SYNTHESIS INHIBITION AND MULTIPLE TRAINING SESSION EFFECTS

New protein synthesis is required for long term habituation, and it appears from Chapter 13 that it is unlikely that amnesia produced from protein synthesis inhibition can be attributed to depletion of constitutively expressed proteins. This suggests that, in long term habituation, new proteins are expressed in a critical period during and shortly after training.

In Chapter 4, retention was evidenced 24 hours after a single training session. Further, multiple training sessions produced long term habituation lasting weeks or months. It could be hypothesised that new, learning specific proteins are synthesised in the critical time window (see Chapters 9, 10 and 13) and these maintain the long term habituation. However, research has demonstrated that neuronal proteins have relatively short half-lives (Barondes & Dutton, 1972) of magnitudes of days or at most, weeks. Thus, degradation processes would reduce modified or newly synthesised 'learning' proteins to half their level within days of training at most. Consequently, it must be assumed that any mechanism responsible for learning involving proteins must be self-replicating, as learning lasts longer than would be possible for individual proteins to survive. Thus, as suggested in earlier chapters, the genome could be involved, and <sup>any</sup> expression of such genes in long term learning must be

maintained; i.e. if new gene expression or changes in gene expression are involved in long term learning, such changes in gene expression must be maintained. Recently, increased attention has focused on gene expression and long term learning (see Chapter 1). Kandel suggests that long term habituation in terms of days, involves the activation of early acting genes, whereas, retention of greater duration involves action on late acting genes with the potential to maintain biochemical changes in neural tissue (see Chapter 1).

In order to test such a hypothesis, a series of further experiments was conducted utilising the ability of long term habituation of the antennae withdrawal response to 'potentiate' with multiple training sessions. That is, greater retention is evidenced when multiple training sessions are presented in series.

The experiments reported in this chapter investigate the ability of learning from an initial training session to survive when subsequent long term habituation from a second training session, is disrupted by various PSIs. If new gene expression is induced during initial learning, and such changes in gene expression are self-replicating, then prior learning established without protein inhibition should transcend subsequent inhibition in one session of a multiple training series to show the multiple training effects on retention established in Chapter 4. Conversely, if new gene expression is not induced, but, proteins synthesised in a second training experience replace biochemical changes already produced in initial training, later protein

synthesis inhibition in a second training session would disrupt replication of the 'initial training proteins' i.e. inhibition during subsequent training sessions would increase trials to habituation to initial training levels. If gene expression is not involved in long term habituation, such a replacement of proteins in subsequent training must occur to account for the time course of retention and 'potentiation' of retention from multiple training sessions (see Chapter 4).

#### METHOD

Three initial experiments were conducted utilising three antibiotics at safe doses (25yg/snail) to investigate the effects of such PSI's on multiple training effects. All experiments were conducted using the following methodology. A final experiment was then conducted using the same methodology but with longer retention intervals.

Subjects. Eighty snails of standard weight were employed in each of the experiments. All snails were captured from the hills of Bovisand Bay, South Devon. Snails were laboratory housed as reported in Experiment 1, Chapter 3.

Procedure. Training procedure, habituation stimulus, and ISI were as reported in Experiment 3, Chapter 4, except all snails received two training sessions interspaced by 12 hours and were retested 12 hrs after the second training session. Each of the three experimental sessions consisted of iterated presentations of a tactile stimulus (Aluminium



prod) until criterion of habituation was achieved. Trials to criterion were recorded exclusive of the three non responses for each of the three sessions. Ten snails were allocated to each of 8 experimental conditions. Pharmacological treatment groups are the same as employed in Chapter 11 and were adapted to this experiment as follows:

TABLE 17.1

GROUP	TRAINING 1	TRAINING 2	12 HOUR TEST
1	DRUG	DRUG	DRUG
2	DRUG	SALINE	DRUG
3	DRUG	SALINE	SALINE
4	DRUG	DRUG	SALINE
5	SALINE	SALINE	SALINE
6	SALINE	SALINE	DRUG
7	SALINE	DRUG	SALINE
8	SALINE	DRUG	DRUG

All injections were given 30 mins prior to the appropriate session and were of volume 0.1 ml. Injection procedure was as described in Chapter 7.

Each of the three experimental sessions was preceded by a 1 minute apparatus orientation period. Failure to emerge in this period terminated a snail's further participation as did snails which withdrew for longer than 1 minute through a trial. Two animals were replaced in Experiment 1, four in Experiment 2, and one in Experiment 3.

## EXPERIMENT 1

### ACTINOMYCIN D INDUCED AMNESIA IN MULTIPLE TRAINING

The above methodology was employed to compare saline and actinomycin D on multiple training effects in long term habituation. The experiment was conducted November 1 to 11, 1986. Laboratory temperature varied from 17 to 19 C.

### RESULTS

TABLE 17.2

MEAN TRIALS TO HABITUATION IN EACH TEST  
FOR EACH TREATMENT GROUP

GROUP	TREAT	TRAIN 1	TRAIN 2 (12 hrs)	RETEST (12 hrs)
1	DDD	11.0	10.9	11.1
2	DSD	11.4	10.7	5.9
3	DSS	10.9	11.0	5.2
4	DDS	10.7	10.8	10.6
5	SSS	11.2	5.3	3.8
6	SSD	10.8	5.6	3.6
7	SDS	11.0	5.4	5.4
8	SDD	10.6	5.0	5.1

TREATMENTS            S = SALINE            D = DRUG

The results are shown in Table 17.2. Analysis of these results in a 2-way ANOVA with a between subject factor of group and a within factor of test (training 1, training 2, and retest) revealed a significant effect of group ( $F(7/72) = 29.919, p < .001$ ) and test ( $F(1/72) = 492.494, p < .001$ ) with a significant group x test interaction ( $F(7/72) = 44.013,$

$p < .001$ ).

Multiple comparisons were then made (following Chapter 12) and revealed in Training 1 there was no difference between actinomycin D treated (Groups 1 to 4) or saline treated (Groups 5 to 8) snails ( $F(7/72) < 1$ ).

In Training 2, 12 hrs after Training 1, Groups 1 to 4 which received PSI in initial training, showed no long term habituation. There was no difference in trials to habituation between these two sessions for these groups ( $F's(7/72) < 1$ ). Further, there was no difference between snails that received drug in Training 2 (Groups 1 and 4) and groups that received saline (Groups 2 and 3) ( $F(7/72) < 1$ ).

Groups 5 to 8, which received saline in initial training did show significant retention over the 12 hrs to Training 2 ( $F's(7/72) = 422.56, 402.38, 403.11, \text{ and } 401.58, p < .001$  respectively). Comparisons between Groups 5 and 6 (drug in Training 2) with Groups 7 and 8 (saline in Training 2) revealed no significant difference in Training 2 scores ( $F(7/72) < 1$ ).

Comparisons of retest scores at 12 hrs post Training 2 showed snails which received drug in Training Session 1 and 2 (Groups 1 and 4) showed no retention at retest ( $F(7/72) < 1$ ). All other groups showed some retention (see results table). Comparisons of this retention revealed a significant difference between groups that received drug in Training 1 and saline in Training 2 (Groups 2 and 3) with groups that received saline in both these sessions (Groups 5 and 6) ( $F(7/72) = 289.638, p < .001$ ). In Groups 2 and 3, there was no retention between Training 1 and 2 but retention at retest.

Groups 5 and 6 showed retention between all sessions, and demonstrated the multiple training effect in retention. There was also a difference at retest between groups which received saline in both training sessions (Groups 5 and 6) and saline, drug groups (Groups 7 and 8) ( $F(7/72) = 266.897$ ,  $p < .001$ ). The saline, drug groups showed retention between Training 1 and 2, but PSI at Training 2 prevented the further retention which was evident in Groups 5 and 6. Thus in saline, drug groups, there was no difference between Training Session 2 and retest scores. Original retention between Training 1 and 2 was not affected.

#### DISCUSSION

Inhibition of protein synthesis by actinomycin D had no effect on short term habituation. Long term habituation was not evidenced in Session 2 where drug was active in Session 1. Further, multiple training effects were disrupted by action of the drugs in either Session 1 or Session 2. Where normal protein synthesis occurred in Session 1, retention was evidenced in Session 2. However, protein synthesis inhibition in Session 2 prevented further saving in a subsequent retest with snails remaining at the same score as evidenced in Session 2. This suggests that earlier learning with normal protein synthesis remains intact and remains impervious to subsequent protein synthesis inhibition once it is established.

## EXPERIMENT 2

### ANISOMYCIN INDUCED AMNESIA IN MULTIPLE TRAINING

The effects of anisomycin on the multiple training session paradigm was also conducted. The experiment was conducted November 12 to 23, 1986. Temperature in the laboratory varied between 18 and 19 C.

### RESULTS

TABLE 17.3

MEAN TRIALS TO HABITUATION IN EACH SESSION  
FOR EACH TREATMENT GROUP

GROUP	TREAT	TRAINING 1	TRAINING 2 (12 hrs)	RETEST (12 hrs)
1	DDD	11.2	12.0	11.6
2	DSD	10.9	10.9	5.6
3	DSS	11.1	11.0	5.4
4	DDS	10.7	10.7	10.6
5	SSS	11.0	5.9	3.2
6	SSD	10.8	5.6	3.5
7	SDS	10.6	5.3	5.8
8	SDD	11.0	5.1	5.0

TREATMENT KEY S = SALINE, D = DRUG

Mean trials to habituation are presented in Table 17.3. These results were again analysed in a 2-way ANOVA and showed a significant effect of groups ( $F(7/72) = 29.637$ ,  $p < .001$ ) and test ( $F(1/72) = 425.221$ ,  $p < .001$ ) with a significant condition x test interaction ( $F(7/72) = 39.106$ ,  $p < .001$ ).

Analysis of simple main effects revealed there to be no

significant difference between groups in Session 1 ( $F(7/72) < 1$ ). There was a significant difference in Session 2 ( $F(7/72) = 57.901, p < .001$ ), and retest ( $F(7/72) = 196.979, p < .001$ ).

Multiple comparisons revealed that in Training Session 1 there was no difference in trials to habituation between drug (Groups 1 to 4) and saline (Groups 5 to 8) treated snails ( $F(7/72) < 1$ ).

In Training 2, groups which received PSI in initial training 12 hrs previous showed no long term habituation ( $F(7/72) < 1$ ). Further, there was no difference between snails that received drug in Training 2 (Groups 1 and 4) and those that received saline (Groups 2 and 3) ( $F(7/72) < 1$ ). Groups 5 to 8, which received saline in initial training, did show retention in Training 2 ( $F$ 's(7/72) = 439.621, 423.964, 419.459, and 432.827,  $p < .001$  respectively). Comparisons between Groups 5 and 6, with Groups 7 and 8 revealed no significant difference between Training 1 and 2 ( $F(7/72) < 1$ ).

Comparisons of retest scores showed groups which received drug in Training 1 and 2 (Groups 1 and 4) showed no retention at retest ( $F(7/72) < 1$ ). All other groups showed some retention (see results Table 17.3). Comparisons of this retention revealed significant differences between groups that received drug in Training 1 and saline in Training 2 (Groups 2 and 3) with groups that received saline in both these sessions (Groups 5 and 6) ( $F(7/72) = 307.087, p < .001$ ). In Groups 2 and 3 there was no retention between Training 1 and 2, but retention at retest. Groups 5 and 6 showed

retention between all sessions and demonstrated the multiple training effect at retest. There was also a significant difference between saline, saline groups (5 and 6) and saline, drug groups (7 and 8) ( $F(7/72) = 299.528, p < .001$ ). The saline, drug groups showed retention between Training 1 and 2, but PSI at Training 2 prevented the subsequent increase of this retention which was evidenced in saline, saline groups.

#### DISCUSSION

These results for anisomycin are the same as those for Actinomycin D.

## EXPERIMENT 3

### PUROMYCIN INDUCED AMNESIA IN MULTIPLE TRAINING

A final experiment used the above design to investigate the effects of Puromycin. The experiment was conducted between November 24 to December 9, 1986. Laboratory temperature varied between 19 and 20 C.

### RESULTS

TABLE 17.4

MEAN TRIALS TO HABITUATION IN EACH TEST FOR  
EACH TREATMENT GROUP

GROUP	TREAT	TRAINING 1	TRAINING 2 (12 hrs)	RETEST (12 hrs)
1	DDD	10.7	10.8	10.6
2	DSD	11.1	10.8	5.0
3	DSS	10.9	11.0	5.8
4	DDS	11.2	10.9	11.0
5	SSS	11.0	5.0	3.1
6	SSD	10.8	5.2	3.2
7	SDS	10.9	5.6	5.3
8	SDD	10.7	5.4	5.1

TREATMENT KEY S = SALINE, D = DRUG

Results for this experiment were analysed in a 2-way ANOVA and revealed significant effects of group ( $F(7/72) = 33.159, p < .001$ ) and test ( $F(1/72) = 464.784, p < .001$ ) with a significant interaction of group x test ( $F(7/72) = 42.980, p < .001$ ).

Further analysis and analysis of simple main effects revealed no significant difference in trials to habituation



in Test 1 between conditions ( $F(7/72) < 1$ ) but a significant difference in Test 2 ( $F(7/72) = 333.972, p, .001$ ) and at retest ( $F(7/72) = 402.996, p < .001$ ).

Multiple comparisons revealed that in Training 1, there was no difference between puromycin treated groups (1 to 4) or saline (5 to 8) ( $F(7/72) < 1$ ).

At Training 2, Groups 1 to 4 which received PSI in Training 1, showed no retention ( $F(7/72) < 1$ ). Further, there was no difference between snails that received drug in Training 2 (Groups 1 and 4) and those that received saline in Training 2 (Groups 2 and 3) ( $F(7/72) < 1$ ). Groups 5 to 8, which received saline in initial training, did show retention at Training 2 ( $F's(7/72) = 400.78, 410.32, 404.11, \text{ and } 409.97, p < .001$  respectively). Comparisons between Groups 5 and 6 (drug in Training 2) with Groups 7 and 8 (saline in Training 2) revealed no significant difference in scores at Training 2 ( $F(7/72) < 1$ ).

Comparisons of retest scores 12 hrs after Training 2 showed snails which received drug in Training Session 1 and 2 (Groups 1 and 4) showed no retention at retest ( $F(7/72) < 1$ ). All other groups showed some retention (see Table 17.4). Comparisons of this retention revealed a significant difference between groups that received PSI in initial training and saline in Training 2 (Groups 2 and 3) with groups that received saline in both these sessions (Groups 5 and 6) ( $F(7/72) = 299.767, p < .001$ ). In Groups 2 and 3, there was no retention between Training 1 and 2, but there was retention at retest. Groups 5 and 6 showed retention between all sessions, and demonstrated the multiple training session

effect at retest. There was also a difference at retest between groups which received saline in both training sessions (Groups 5 and 6) and saline, drug groups (7 and 8) ( $F(7/72) = 280.663, p < .001$ ). The saline, drug groups showed retention between Training 1 and 2, but PSI at Training 2 prevented the increase in retention which was evidenced in Groups 5 and 6.

#### DISCUSSION

Again, these results are the same as those reported for actinomycin D and anisomycin.

#### EXPERIMENT 4

A final experiment was conducted using one PSI to investigate multiple training effects over longer periods of time. In this experiment the PSI anisomycin was employed, Training Session 2 was 24 hours after Training Session 1, and retest was 72 hours after Training Session 2. The Experiment was conducted March 1 to March 19, 1987. Temperature in the laboratory varied from 13 to 19 C.

## RESULTS

TABLE 17.5

MEAN TRIALS TO HABITUATION IN EACH TEST FOR  
EACH TREATMENT GROUP

GROUP	TREAT	TRAIN 1	TRAIN (24 hrs)	RETEST (72 hrs)
1	DDD	11.2	10.7	11.1
2	DSD	11.1	11.2	11.3
3	DSS	10.8	11.0	10.6
4	DDS	11.5	10.8	10.6
5	SSS	11.3	5.4	4.1
6	SSD	10.9	5.6	4.3
7	SDS	11.0	5.4	9.9
8	SDD	10.9	5.0	11.3

Mean trials to habituation are reported in Table 17.5. Analysis of these results in a 2-way ANOVA with between subject factors of condition and test revealed a significant effect of group ( $F(7/72) = 19.856, p < .001$ ) and test ( $F(1/72) = 196.348, p < .001$ ) with a significant interaction of group x test ( $F(7/72) = 12.749, p < .001$ ).

Further analysis and analysis of simple main effects revealed no significant difference in trials to habituation between groups in Training 1 ( $F(7/72) < 1$ ) but a significant difference in Training 2 ( $F(7/72) = 639.854, p < .001$ ) and at retest (72 hrs) ( $F(7/72) = 674.341, p < .001$ ).

Multiple comparisons revealed that in Training 1, there was no difference between anisomycin treated groups (Group 1 to 4) or saline (Groups 5 to 8) ( $F(7/72) < 1$ ).

At Training 2, however, Groups 1 to 4 which received PSI in Training 1, showed no retention ( $F(7/72) < 1$ ). Further, there was no difference between snails that

received drug in Training 2 (Groups 1 and 4) and those that received saline in Training 2 (Groups 2 and 3) ( $F(7/72) < 1$ ). Groups 5 to 8, which received saline in initial training, did show retention at Training 2 ( $F's(7/72) = 399.52, 387.62, 385.94, \text{ and } 400.71, p < .001$  respectively).

Comparisons between Groups 5 and 6 (drug in Training 2) with Groups 7 and 8 (saline in Training 2) revealed no significant difference in scores at Training 2 ( $F(7/72) < 1$ ).

Comparison of retest scores 72 hours after Training 2 revealed retention was only evidenced in Group 5 (saline, saline, saline) ( $F(7/72) = 49.93, p < .001$ ) and Group 8 (saline, saline, drug), i.e groups which were unaffected by PSI in either training sessions. All other groups, which had received drug in either Training 1, or Training 2, or in both showed no retention from training 2 to retest ( $F(7/72) < 1$ ). Similarly, there was no difference in retest scores between groups which received drug in Training 1, but not in Training 2 (Groups 2 and 3), and groups that received drug in in both Training 1 and 2 (Groups 1 and 4) ( $F(7/72) < 1$ ). Retest score comparisons also showed no difference between Groups 2 and 3, and those receiving saline in Training 1, and drug in Training 2 (Groups 7 and 8) ( $F(7/72) < 1$ ), and no difference between drug in retest (Groups 1, 2, 6, and 8) with groups with saline in retest (Groups 3, 4, 5, and 7) ( $F(7/72) < 1$ ). Similarly, further evidence that the drug had no effect at retest was provided by retest score comparison between Groups 5 and 8 ( $F(7/72) < 1$ ).

## DISCUSSION

If protein synthesis was intact during initial training or Training 2 there was retention over 24 hours. However, as Groups 2, 3, 7, and 8 show, retention was not maintained over 72 hrs without a second PSI-free training session. This was the case even when retention of learning from initial training was expressed in second training session under the influence of the PSI.

### GENERAL DISCUSSION

The initial three experiments demonstrate that protein synthesis inhibition has no effect on short term habituation but does disrupts long term habituation when synthesis is inhibited during or shortly after training. Further, in the multiple training session design, learning which occurred with normal protein synthesis remained intact, and subsequent inhibition did not disrupt such earlier established long term habituation.

Retention from initial training can survive subsequent protein synthesis inhibition in a second training session, but such retention sampled at a further retest is not increased by the second training. Thus, the result suggests that once long term learning becomes established this cannot be disrupted by PSI, but the process by which this retention becomes potentiated can be disrupted. However, the results of Experiment 4, using longer intersession intervals showed that the initial retention evidenced at 24 hours, could not

survive without a subsequent training session which was also protein synthesis free. Thus in this experiment only snails that had received no drug in either training sessions showed any retention in the 72 hour test. Also if protein synthesis was disrupted in initial training, retention from protein synthesis intact Training 2 could not survive.

Such results are in accord with a hypothetical mechanism of long term habituation involving interaction of the genome. Further, retention between sessions require new protein synthesis and attenuation of retention also requires new protein synthesis.

The results raise the question of how multiple training effects occur. It could be suggested that, in the initial training, new gene expression codes for proteins which are required for the learning. However, how is such expression potentiated to produce the greater retention which was evidenced from two training sessions? It has recently been suggested that such long term learning induced gene expression may involve more than one process; namely, an initial gene expression, which, with subsequent training, induces a more stable, secondary cascade expression phenomenon; i.e early and late effector and regulator genes (Goelet et al., 1986; Goelet & Kandel, 1986; Ray, 1987). The concept of gene cascade systems has evolved from research on gene expression in early embryos and has been suggested to account for the stages of retention evidenced in in-vitro learning in *Aplysia*. This will be discussed in the final chapter.

From this in-vitro model of simple learning, Kandel and

colleagues have suggested a mechanism of short term habituation dependent on phosphorylation of pre-existing proteins by a secondary messenger c-AMP. Retention in the magnitude of days is suggested to be the product of induction of new proteins and such induction is attributed to c-AMP, which modify trans-acting regulators that activate both 'early effectors' and 'early regulator' genes. Kandel suggests that such learning is retained for the half-life of the effector proteins of such effector genes. He argues retention over periods longer than this is produced by induction of early regulator genes. The protein products of such genes trigger maintained expression of late effector genes. These hypothetical mechanisms are more fully described in Chapter 1.

The results of the experiments reported in this chapter could support such a multi-process model of memory stages as put forward by Kandel. Retention between Training Sessions 1 and 2 could be the result of induction of early genes in initial training, and the product of this expression may account for retention to Session 2 by utilising the proteins half-life, thus, such retention would require initial intact protein synthesis but would not be disrupted by subsequent inhibitions. If PSIs are active in Session 2, retention is not increased to a normal multiple training session effect which produces more robust lasting retention, possibly by interference with the the half-life proteins further interaction with gene expression.

Experiment 4 would support such a view in that initial retention of the magnitude of 24 hours could survive by

protein half life of the product of initial, induced, protein synthesis. But, over greater retention intervals (e.g. 72 hours), the initial proteins cannot survive, thus to produce longer retention, a second protein synthesis dependent process is required perhaps to produce a maintained alteration in gene expression. Such a model will be further discussed in the final chapter.



DISCUSSION: THE EFFECTS OF PROTEIN SYNTHESIS INHIBITION  
ON SHORT AND LONG TERM HABITUATION OF THE DORSAL  
ANTENNAE WITHDRWAL RESPONSE OF HELIX ASPERSA

Habituation has proved a useful learning behaviour for an investigation of the effects of protein synthesis inhibition on short and long term learning. Results demonstrate that short term habituation does not require protein synthesis, but that learning induced protein synthesis is an integral component of the mechanism of long term habituation. This final chapter extends the general discussions included at the end of each chapter and serves to link some of the results which initially may have been discussed in isolation.

A prerequisite for progress in this area is the establishment and behavioural understanding of a suitable learning behaviour. Indeed, an important criticism of the area has been the failure to satisfy this prerequisite. This problem has been exacerbated by the piecemeal manner in which studies have used vastly different learning situations, animals, and methodologies to demonstrate different characteristics of PSI effects on learning. Thus, a coherent and comprehensive investigation of PSI effects in an intact animal is conspicuous in the literature by its absence. Consequently, the initial section of the thesis strived to establish a suitable, simple learning behaviour in a simple system which would be readily reproducible both

within and between laboratories. Habituation of the dorsal antennae withdrawal response in the snail Helix aspersa appears to fulfill the above criteria.

The early chapters of the thesis established that the response decrements to iterated stimulus presentations, and that such a decrement could be obtained with a variety of stimuli (see Chapter 3). Having tested the habituation methodology and response measurement employed for experimenter effects and inter-observer reliability, the decrement in response was then established as habituation rather than sensory fatigue in a series of experiments that demonstrated a change stimulus and dishabituation effect (Groves & Thompson, 1970; Thompson & Spencer, 1966). The change stimulus effect was established using a variety of habituation stimuli.

The subsequent behavioural research demonstrated that habituation in the snail is comparable to vertebrate organism habituation.

Invertebrate learning has sometimes been regarded as a temporary phenomenon (see Chapter 4), and, as a result, its relevance to vertebrate learning questioned. Research presented here suggests that habituation in the snail is far from a temporary phenomenon. Learning is retained for up to 24 hours after one session of training to criterion. From two training sessions presented in series, retention is evidenced at 3 weeks post training and from 10 training sessions habituation showed retention at 6 months post training. This demonstration also served to establish this response decrement as habituation rather than fatigue.

Research in Chapter 4 also highlighted the relative advantages and disadvantages of fixed number of trials and trials to habituation paradigms and the value of multiple measures of habituation was discussed. All subsequent research was demonstrated with both habituation paradigms.

Habituation is a particularly interesting form of behavioural plasticity for investigation of protein synthesis inhibition. Behavioural evidence concerning the parametric characteristics of this form of learning suggests that short and long term habituation are mediated by different processes (see Chapter 2). This has particular consequence for recent theories of habituation. Arguably the most important evidence pertaining to this issue is the effect of massing and spacing of training on short and long term habituation and, in particular, the effects of ISI. Research has suggested that short ISIs produce faster short term habituation, but that this is not the case for long term habituation. Evidence from Helix presented in Chapter 5 supports this conclusion in that short ISIs produced short term habituation with fewer trials, and there was less recovery between stimuli evidenced by responses of smaller durations and magnitudes. In long term habituation this superiority of small ISIs was not found. Research reported in Chapter 6 extends evidence of differential short and long term processes by investigating other forms of massed and spaced training and demonstrated that within session spacing over longer intervals than used in the ISI chapter had no effect on trials to habituation in short term habituation or the development of such habituation compared to massed

training. Conversely, this spaced training showed greater retention at a variety of retest intervals. These results are in accord with the more extensive vertebrate literature. The differential effects on short and long term habituation would support theories which posit dual processes for the two phenomenon. The lack of differential development of habituation in training reported in Chapter 6 is particularly interesting in that there was superior retention of habituation from spaced training, but the inter block interval did not evidence greater recovery between the last response of one block to the first response of a subsequent block compared to equivalent responses in massed training. This would intimate that although an observable response recovery was not evident behaviourally, some recovery could have occurred at the physiological level. Further research utilising greater intervals would be of great interest in light of these results.

Using the behaviours established in the earlier chapters, the final 12 chapters of the thesis address the suggestion of protein synthesis involvement in both short and long term habituation.

Having established a suitable injection technique and determined the time of biochemical action of the drugs to be employed (see Chapter 7), the second major problem of PSI research was addressed. That is, the potentially confounding side effects and general behavioural debilitations of these powerful pharmacological agents.

Researchers have tried several ways of countering this criticism (see Chapter 8), but the problem has remained.

Consequently, a battery of six behavioural tests was designed and developed (Chapter 8) to demonstrate that given doses of particular PSI are side-effect or debilitation free. This work established safe doses of three protein synthesis inhibitors which had been demonstrated to inhibit protein synthesis in snail neuronal tissue after a pedal injection (see Chapter 7). The battery of tests developed also help to counter alternative explanations of PSI effects, however, abnormal catecholamine synthesis (Barondes & Cohen, 1968), adrenal steroidogenesis (Nakajima, 1975), and elevation of cerebral free amino acids (Goodman et al., 1975; Spanis & Squire, 1978), cannot be ruled out. Nevertheless other hypothetical mediators of the amnesic effects should have altered at least one of the six behaviours employed in the battery of tests. For the same reason, the suggestion that antibiotics at amnesic doses produce sickness, locomotor deficits and abnormal responses was also rejected.

Three antibiotics, at four doses, were found to meet criterion for further inclusion in learning studies, anisomycin, actinomycin D and puromycin. Cycloheximide was found to have a debilitating effect at all doses employed and, therefore, was not used in the rest of the thesis.

The test battery developed here could usefully be adapted for testing of other pharmacological agents economically and serve as a heuristic for the development of such a battery for inclusion in other PSI studies in different animals.

Safe, side-effect free, doses of anisomycin, actinomycin D, and puromycin were then investigated for

their effects on short and long term habituation. Short term habituation was not affected by anisomycin, actinomycin D, or puromycin at doses under 25yg/snail. The same doses, however, disrupted long term habituation when active within a critical time window which was during and briefly after training (see Chapter 9). Research then went on to show that the disruption of long term habituation could not be attributable to the PSIs having either short or long latency performance effects or to any state dependency effects (see Chapter 10). Further, comparisons made between experimental conditions in Chapters 10 and 11 showed that multiple injections of antibiotics did not produce greater amnesia than that evidenced by snails which had received one injection. It could be argued that if injections of antibiotic were debilitating the snails in retest, then snails which had received a double dose of drug would show such debilitation to a greater extent. Similarly, these comparisons suggest that the drugs prevent synthesis of learning specific proteins. If the amnesic effect was due to general protein depletion, greater depletion would have occurred in multiple injection conditions and result in a greater effect in long term habituation. This was not found.

One recent suggestion has centred on evidence from goldfish that the amnesic effect observed with PSIs may be due to stress (see Chapter 12). Comparisons of PSI effects in a variety of high and low stress experimental situations found the same amnesic effect of PSIs in both conditions and, further, demonstrated that intact short term habituation could still show a change stimulus effect even

in the presence of PSIs (see Chapter 12).

Research presented in Chapter 13 went on to investigate the critical time window for protein synthesis suggested by research in earlier chapters. Injection time was found to be critical for PSI induced amnesia and was, to some extent, dependent on dose. All experiments demonstrated, however, that protein synthesis was necessary during or immediately after training for long term habituation to be evidenced. It is interesting that injections more than 80 min prior to training had no effect on long term habituation. This suggests that a proportion of the inhibitory effect of the drug has worn off by the critical time period. Injections after 40 minutes post training, again did not show any amnesic effect in long term habituation. Given that these antibiotics are known to inhibit protein synthesis, that this effect lasted between two and two and a half hours dependent upon dose (see Figures 7.2 to 7.4), and that these drugs act almost immediately, it could be suggested that, in the snail, protein synthesis must remain uncompromised during initial acquisition of learning and for approximately 40 minutes after learning for long term habituation to occur. Thus, biochemical evidence from labelling techniques, and behavioural evidence from time of behavioural effect analysis are in agreement.

Further, the findings that these early injection times for PSIs do not affect long term habituation must question the suggestion that PSI induced amnesia is the product of a depletion of constitutive proteins. These early injected snails would still have undergone protein synthesis

inhibition which would have disrupted their supply of necessary constitutive proteins and should, therefore, have produced amnesia to some extent if these constitutive proteins were involved in long term retention. This will be discussed later.

The above results show that short term habituation in the intact snail was protein synthesis independent, and long term habituation was protein synthesis dependent.

The research reported above is of relevance to a variety of theoretical issues concerning the effects of PSIs and the biological basis of learning. These will now be examined.

#### 18.1 SHORT TERM HABITUATION

Research in vertebrates suggests that protein synthesis inhibition is not required in short term learning (Agranoff, 1968; Barondes, 1975; Barondes & Cohen, 1968a; Cohen & Barondes, 1968; Squire & Barondes, 1974; Flood & Jarvik, 1975; Squire, 1975). This has also been demonstrated in an in-vitro model of short term learning in invertebrates (Schwartz, Castellucci, & Kandel, 1971), and the results presented in chapters 9, 10, 11, 12, and 13 agree with these findings. Indeed, throughout the PSI chapters, there was never any observable effect of protein synthesis on short term habituation.

This lack of PSI effects on short term learning has been traditionally demonstrated by comparing performance on acquisition of a learning task with and without protein



synthesis. To extend this work, experiments reported in Chapters 14, and the final experiment of Chapter 12, demonstrated that three PSIs with different modes of inhibition have no effect on the relationship between ISI and short term habituation. Short ISIs produced habituation to criterion with fewer trials than long ISIs in the absence and presence of protein synthesis (see Chapter 14). Further, during protein synthesis inhibition, the response could be re-evoked by a change stimulus which suggests that short term processes were not affected by PSIs (see Chapter 12).

One suggested effect of PSIs on short term learning has been the disruption by PSIs of later trials during acquisition. (Gutwein et al., 1974; Quartermain & McEwen, 1970; Rainbow et al., 1976 and Randt et al., 1971). Experiments reported in this thesis which chart the development of short term habituation in terms of both response magnitudes and durations found no such effect.

Another suggested short term effect of PSIs has been the prolongation of short term retention (Quinton, 1978). However, research reported in Chapter 15 demonstrates normal recovery of response from short term habituation after training in the absence of protein synthesis. One of the most interesting results of work in *Helix* was that retention was not affected up to 1 hr post training by the PSIs. This suggests that the short term mechanism remained intact despite the inhibition, and that short term habituation does show some transient retention, even when long term habituation is disrupted by protein synthesis inhibition.

The above results are supportive of theories of a short

term learning mechanism that does not require protein synthesis. The most extensive research on a short term mechanism of habituation remains that of Kandel and colleagues who reported evidence for phosphorylation of pre-existing membrane proteins by cellular secondary messenger cAMP (see Chapter 1). Certainly, evidence reported in this thesis conclusively demonstrated that the mechanism of short term habituation in the snail does not require new protein synthesis and could therefore involve post-translational modifications of stable macromolecules of which proteins remain an ideal candidate. It should, however, be considered that proteins can be post-translationally modified by a variety of secondary messengers.

PSI research can do little to further expand upon potential mechanisms of short term processes. However, it is hoped that a conclusive demonstration of such an effect serves as a useful heuristic in the elucidation of short term mechanisms. Further, the research on ISI, intact ISI effects when protein synthesis is inhibited, and some evidence of retention for between 1 and 2 hours when PSIs have been active, will be of value in this endeavour.

The lack of any requirement for protein synthesis in short term habituation and its retention in the snail bears upon theories which have suggested distinct stages of memory formation. However, before discussing multi-stage theories of habituation, let us further consider the effects of PSIs on long term habituation.

## 18.2 LONG TERM HABITUATION

As has been demonstrated in Chapter 4, retention of habituation is evidenced at 24 hours after one training session to habituation, and this retention is strengthened by repeated series of training sessions with evidence of retention for up to six months.

Research on the disruption of long term learning by PSIs has traditionally followed the methodology discussed in the short term habituation discussion (Section 18.1); namely, measurement of retest performance, time to reacquisition, and savings in retrials. The employment of such measures in the snail again reveals PSIs disrupt long term habituation. This conclusion is further extended by investigating the parametric characteristics of habituation demonstrated in the retest. Research in Chapter 16 reveals that protein synthesis inhibition in the critical time window established in Chapter 13, causes complete amnesia of the long term habituation in that, at retest, the re-habituation shows the parametric characteristics associated with short term habituation rather than long term, i.e. the trained, PSI treated snails behave as though they had received no prior training. This completeness of amnesia was demonstrated by PSI treated trained snails showing a short term ISI effect in the long term retest. Indeed, this demonstration of the parametric characteristics of short term habituation where long term characteristics should be in evidence further supports the conclusions of chapter 8. That is, the PSIs were obviously not debilitating

the snails in retest, and, perhaps more interestingly, the mechanism of reacquisition of learning shown by these PSI treated snails at retest was not disrupted by their early pharmacological manipulation.

The above PSI induced amnesia was demonstrated to be a robust phenomenon by repeated replications of this main effect, and by demonstrations of this effect using three different antibiotics, and a variety of doses. Research in Chapters 7 to 17 shows a remarkable similarity between these drugs in terms of their main effect, time of effect, and inhibitory actions. Only slight differences between drugs were found in the time of their effects and the dose/effect relationships. Each of these antibiotics disrupts protein synthesis by a different mode of action, but all were capable of disrupting long term habituation. Also, their mode of actions suggest that long term habituation requires intact transcription and translation, as Actinomycin D, a powerful mRNA inhibitor, also produced amnesia as well as puromycin and anisomycin both of which impeded protein synthesis at the translational stage. Similarly these two processes are required at approximately the same time during, or for a brief period after, training.

Research in Chapter 13 demonstrates the critical time period when protein synthesis is required for long term habituation. As stated above, the three antibiotics showed consistency as to when this period occurred in relation to training. Chapters 10, 11, and 12 all demonstrate that inhibition of protein synthesis immediately prior to retest has no amnesic effect, if protein synthesis has remained

intact prior to this. Chapter 13 extended this finding to investigate the actual time period of disruptive action of the antibiotics at three different doses.

The results support those found with other forms of learning. The critical period for protein synthesis is during and for approximately 40 minutes after training. Protein synthesis disruption prior to training, provided the effects of the drug have stopped (i.e. two to two and a half hours) (see Chapter 7) does not disrupt long term habituation. It has been argued that such an effect is evidence that PSI induced amnesia is not the product of a depletion of constitutive proteins (e.g., Dunn, 1980; Squire & Barondes (1976), Squire & Davis, 1984) (see Chapter 13).

While the data presented in Chapter 13 would support such a conclusion, it must be qualified by the lack of knowledge of protein half-lives in snail neuronal tissue. It is possible that two hours protein synthesis inhibition is not a sufficient duration to significantly deplete necessary constitutive proteins. However, that drug injection times of greater than 80 minutes prior to training failed to produce any amnesia, from any dose of any of the three antibiotics, does suggest that the amnesia was not the product of a depletion of constitutive proteins. Constitutive or existing protein modification as a mechanism of long term learning appears to be an unlikely mechanism of very long term retention. Theories providing a mechanistic account of such processes will be discussed in a later section (see section 18.4). The results of the thesis do suggest that new protein synthesis is required for long term habituation, and this

would implicate changes in gene expression. One hypothesis could be that post-translational changes of pre-existing or newly synthesised proteins could be replicated by interaction with the genome.

The durability of retention of habituation in the snail (see Chapter 4) would suggest that changes in protein synthesis must be maintained, for example, by changes in gene expression. If learning dependent proteins can provide retention past the recognised half-lives of neuronal proteins, the synthesis of such molecules must be coded. Thus, a series of experiments are reported in Chapter 17, which serve to investigate and advance such a hypothesis by studying the effects of PSIs on very long term habituation after evidence of an initial retention. This work utilises the finding that multiple training sessions provide more durable retention.

By inhibiting protein synthesis at various stages of a series of training sessions and subsequent retest, the initial three experiments reported in Chapter 17 show that if training in session 1 occurs with protein synthesis intact, retention is evidenced 12 hours later. Similarly, a second protein synthesis intact training session increases retention evidenced in a retest after a further 12 hours. Interestingly, if protein synthesis was inhibited in Session 2 after initial training was protein synthesis intact, the saving in trials to habituation at Session 2 survived the subsequent PSI treatment to manifest itself at retest. This retest retention was, however, not as great as that exhibited by snails that had received two protein synthesis

intact training sessions. Thus, any potentiating effects of training 2 were abolished by PSI, but original retention survived. This was found with all three antibiotics used. A further experiment investigated this phenomena over longer time intervals. This demonstrated that there was retention from one PSI-free, training sessions at 24 hours. However, for retention to be evidenced at 72 hours after a second training session, protein synthesis had to remain intact in both training sessions, without a second protein synthesis intact session, retention could not survive 72 hours. This result suggests that long term habituation may, infact, involve more than one process, and these processes require the synthesis of new proteins. Further, the results of Chapter 17, make the demonstration of intact retention for 1 hour even when protein synthesis was disrupted very exciting. It supports a multi-process point of view in habituation and has bearing on other multi-process theories of learning.

### 18.3 STAGES OF MEMORY FORMATION AND PROTEIN SYNTHESIS INHIBITION

The above results could be construed as indicating either two or three stages of habituation. Two stage theories of learning and memory became increasingly prevalent in the mid 1970s due to studies of ECS which showed a sensitive and non sensitive stage of learning (see Gold & McGaugh, 1975). ECS studies, like those using PSIs, have served to differentiate stages of susceptibility but

not the time courses of stages of learning and retention. Two results from this thesis will be discussed in this context; first, the intact retention evidenced from protein synthesis intact short term habituation; second, the time of retention evidenced by protein synthesis dependent learning.

It has been suggested that retention from protein synthesis independent short term learning processes may serve as a measure of the duration of such processes (Barondes & Cohen, 1967b). These authors report retention in mice persists for 3 to 6 hours after discrimination learning. Similar retention for approximately 90 minutes has been reported after passive avoidance training in the chick (Mark & Watts, 1971), and for 1 hour after short term habituation of the dorsal antennae withdrawal response in the snail (Chapter 14). However, such conclusions may be premature, as performance after training may not solely depend on the short term process. It could be argued that towards the latter stages of training long term habituation processes have an effect.

It is known that PSIs do not offer 100% inhibition of protein synthesis. Squires & Barondes (1972) have suggested that the small residual capacity to synthesise proteins could permit a weakly formed long term learning. This suggestion is, in part, based on the observation that amnesia sometimes develops very gradually after training (e.g. Flood et al., 1972). This was not the case in the snail (see Chapter 15). Further, there was no difference between different doses of PSI on short term habituation or its retention. If residual protein synthesis accounted for



retention, higher doses would have produced less retention than low doses. A dose effect was also absent in studies reported by Watts & Mark (1971a, 1971b) who reported no difference in short term retention when PSI dose was increased ten fold. It seems unlikely therefore that retention of short term learning is due to any residual long term process, but is, indeed, a manifestation of a separate protein synthesis independent process.

Having provided evidence in support of stage theories of learning, results from this thesis are also of relevance in deciding between two stage or three stage theories as proposed by Gibb and her colleagues (Gibb & Ng, 1980), Dudai and his co-workers, (Dudai & Quin, 1980), and from Kandel's laboratory (Montarolo et al., 1986).

Gibbs proposes a theoretical mechanism for each of three stages of learning. An initial short term phase is said to depend on neuronal hyperpolarization resulting from increased potassium conductance following neuronal activity. A second, labile stage, is dependent on sodium pump activity, and a final stage is thought to require protein synthesis during "or near" the time of initial training.

From evidence in *Aplysia*, Kandel and colleagues originally viewed habituation as a two process phenomena. A short term process was said to be subserved by post-translational modification of pre-existing proteins by the secondary messenger cAMP, and a long term protein synthesis dependent process was thought to be the result of cAMP phosphorylation of Histone proteins to induce new gene expression (Kandel & Schwartz, 1982). Recently, they have

extended this two phase model to a three phase model (Goellet et al., 1986). In this, they still suggest short term modifications of membranes by post-translational modifications as a mechanism of short term habituation but now include two protein synthesis dependent stages: An initial long term process capable of retention in terms of days which requires the expression of genes but is limited by the half-life of the resultant molecule, and retention in terms of weeks or longer that is subserved by a stable self-maintained change in gene expression (see Chapter 1).

Research, particularly in the later chapters of this thesis, extend the initial finding that short term habituation is protein synthesis independent and long term habituation is protein synthesis dependent and provides some support for the involvement of a third phase similar to that suggested by Kandel.

Evidence from Chapter 17 shows that protein synthesis dependent retention is evident after one training session for up to 24 hrs, but it does not last over longer retest intervals. However, when snails receive two training sessions, retention is evidenced 72 hours post training, and behavioural investigations show that multiple training sessions are capable of showing retention over much greater periods (see Chapter 4). For such multiple training effects to occur protein synthesis must remain intact in both training sessions (see section 18.2).

This area remains clouded by a problem of definition as to what constitutes short and long term habituation. But, habituation of the dorsal antennae withdrawal response in the

snail Helix aspersa may be a three stage process, with at least three independent mechanisms.

Short term habituation of magnitudes of seconds to 1 hour are mediated by protein synthesis independent changes in neuronal function. PSI investigations can do little to investigate such mechanisms, but the data presented in this thesis could conceivably be consistent with the kind of mechanism proposed by Kandel.

From this, it could be argued that long term habituation can be divided into two, protein synthesis dependent processes. First, there is retention of up to 24 hours which requires new protein synthesis, but not retained over longer periods, but that can survive subsequent PSI manipulation (see Experiments 1, 2, 3, Chapter 17). Second, there is a further protein synthesis dependent process that requires multiple, protein synthesis intact training sessions.

It may be that the initial long term retention of the magnitude of 24 hours could be subserved, as Kandel suggests, by the half-life of an induced protein, but that if this retention is to be extended, the original protein product of the training must be re-synthesised. This would require an initial change in gene expression to become maintained. There are at least two ways in which this might work. The protein product could reinforce a change in membrane function to alter the reflex pathway physiology, and consequently, in a second training session, the cell would be at a different physiological state; presumably conducive to activation of self-maintained gene expression.

Alternatively, the initial protein product could be a regulator protein which, if continually synthesised in a second training session may initiate a self-maintained change in gene expression that could alter the physiology of the cell and subsequently the pathway. In such models, the protein product of the self-maintained gene expression changes could interact with membrane changes suggested as responsible for short term habituation (Goelet et al., 1986). An important point that is often overlooked in studies of long term learning mechanisms is that in retest a short term mechanism also occurs as the learning becomes re-acquired.

#### 18.4 SPECULATIONS ON MECHANISMS OF HABITUATION

Although PSI investigations can suggest that protein synthesis is required for long term habituation, they are limited in determining how such proteins could be involved in changing neuronal physiology and, ultimately, behaviour. However, the results of behavioural and pharmacological manipulation of habituation in the snail reported in this thesis do encourage speculation on how new proteins could facilitate retention of learning.

Research in the thesis suggests a three phase process of habituation. The protein synthesis dependent process revealed by the PSI studies could be accounted for by a number of biochemical changes possible in the cell. The work does suggest that long term habituation is accompanied by changes in gene expression and that these changes may become

self maintained. Two cellular mechanisms conducive to maintained changes are discussed. First changes in pre-existing molecules by post-translational modification, second, changes in gene expression.

#### 18.4.1 CONSTITUTIVE PROTEINS, AND PROTEIN SYNTHESIS INHIBITION

The amnesic effect observed in long term learning with PSIs has been attributed by some authors to the product of a depletion of constitutively expressed proteins, or post-translational modification of pre-existing proteins (see Chapter 13).

Crick (1984) has suggested that acquisition of learning involves the activation of pre-existing proteins by covalent modifications. He goes on to suggest that retention relies on a self-reinforcing mechanism which outlasts the turn over time of constituent molecules. His proposed mechanism involves the shift of a dimer to an active state where both subunits are modified, perhaps by a cytoplasmic secondary messenger. Thus, it could be hypothesised that this active state could be retained despite turnover of a single subunit, by an enzyme which can modify one monomer only when the other is modified.

While this theory is speculative, a similar theory (Lisman, 1985) has received some empirical support. Lisman proposed that competing activities of an autophosphorylating kinase and an associated phosphatase make up a 'bistable switch'. Learning stimulates the phosphorylation of the

kinase and thereby initiates its autophosphorylation. When the rate of autophosphorylation exceeds that of dephosphorylation, phosphorylation of the kinase becomes independent of the stimulus provided by learning. Empirical support has been derived from studies on the Calcium ion/calmodulin protein kinase in which intramolecular autophosphorylation permits persistent activation of the kinase in the absence of Calcium ions (Saitoh & Schwartz, 1985; Miller & Kennedy, 1987). However, for this model to be independent of protein turnover either autophosphorylation must be intermolecular or subunit exchange must take place (Goelet et al., 1986). Neither has yet been demonstrated.

A further model of learning based on constitutive protein modification has been suggested by Lynch and Baudry (1984). It is based on studies of long term potentiation. They suggest a model involving the self-reinforcing disassembly of molecular assemblies. They reported that, during acquisition, calcium ion influx produced by learning rapidly and irreversibly increased the number of glutamate receptors in the synaptic membrane by activating calpain, an enzyme that degrades fodrin, a structural protein in the post-synaptic cell. This degradation is proposed to be the means whereby changes in activity in the post-synaptic cell lead to the insertion of new glutamate receptors which, in turn, enhance calcium ion entry and thus generate the reinforcement necessary for the maintenance of the learning.

The three contemporary constitutive protein or post-translational modification based models, have difficulty in account for the requirement of new protein

synthesis in long term learning demonstrated in this thesis and by other researches (see Chapter 1). However, it could be speculated that post-translational modification of existing proteins would require the use of enzymes and this requires protein synthesis. However, such changes would have to become a stable feature of the cell if the modified protein was to survive longer than its own half-life or that of its constituents. Thus, such a model would find difficulty in interpreting the data from the multiple training effects chapters. It is difficult to explain the strengthening of retention by multiple training sessions unless more proteins were modified. An increase in the number of molecules changed, however, does not increase the protein half-life, and it is unlikely that such changes could persist for six months (see Chapter 4). Furthermore, the time of effect analysis (see Chapter 13) demonstrated that injections of antibiotics two hours prior to learning, at four different doses, had no effect on either short or long term habituation of the response. In these experiments early injection times would have depleted constitutive proteins.

Evidence in this thesis does not, however, preclude such changes as a component process in long term habituation, but both the biochemical and behavioural data point to a change at the genome. Such a change may, indeed, facilitate a change in a post translationally modified protein. Such a mechanism could account for the two protein synthesis dependent processes suggested in Chapter 17. To consider gene expression models, and post-translational

modification of constitutive protein models as mutually exclusive may be both premature, and detrimental to our understanding of the mechanisms of learning and memory.

#### 18.4.2 GENE EXPRESSION AND LONG TERM LEARNING

Recently, a plethora of theoretical speculation has emerged suggesting that long term learning does require the novel expression of genes (Goelet et al., 1986; Kandel & Schwartz, 1982; Montarolo et al., 1986; Thompson et al., 1987). These have received scant empirical investigation in terms of behaviour, with such evidence as there is being derived from models of learning in re-aggregate cells and from evidence of induced gene expression in other physiological systems (see Chapter 1).

Kandel and his colleagues have suggested a molecular stage theory of short and long term retention (see Chapters 1 and 17) in which short term learning is mediated by secondary messenger induced phosphorylation of existing membrane proteins, and longer retention is mediated by action at the genome. Based on evidence that secondary messengers such as cAMP are potent gene activators in other systems (Nagamina & Reich, 1985), Kandel et al. have suggested that retention of magnitude of hours to 1 day may be mediated by induced expression of an 'early effector' or 'regulator' gene and is limited by the half life of the resultant protein product. Longer retention is produced by the early regulator genes' proteins triggering maintained expression of late effector genes. Such a model is in



keeping with the three stage process of habituation suggested by research in this thesis.

Kandel's concept of 'early' genes acting as regulators for the expression of 'late' effector genes is a concept borrowed from the molecular biology of embryonic development and, more specifically, cellular differentiation. Goelet et al., quote the evidence of Richards & Ashburner (1984) who demonstrated that the ecdysone mediated induction of growth and moulting in insects is the product of altered 'early' gene expression whose induction regulates the expression of late genes. Richards et al. found that if ecdysone was given with a PSI, the early genes are induced, but the late ones are not. However, if the inhibitor is injected several hours later, the late genes are induced normally. This critical time window effect was found in snail learning.

Early and late acting genes have been more extensively studied in adenovirus in which the identified nuclear oncogene E1A, an early gene, also regulates the expression of the late adenoviral genes. Thus, the basic premise of an early gene affecting a late gene is viable, but how such genes are expressed and this expression is maintained must be further explored. A useful advance in this area would be the identification of nuclear signals that communicate between early and late acting genes.

However, theories which suggest that early mechanisms of retention need subsequent processes induced by subsequent experience could be questioned by the enduring nature of some one trial learning and imprinting. Such learning paradigms may be more conducive to early gene expression

having a more robust resistance to decay or a rapid activation by early genes of late acting genes.

A further point of relevance to such gene expression models of long term learning, is the evidence of prolific gene expression in neuronal tissue compared to less plastic organs. Recent advances in molecular biology have begun to pay dividends in our understanding of neuronal gene expression. By reverse transcribing expressed mRNA to DNA (cDNA) via reverse transcriptase, Sutcliffe and his colleagues have demonstrated that genes expressed specifically by the brain in a rat account for the vast majority of the 30,000 mRNA isolated from the rat brain (Sutcliffe, & Milner, 1984). From this, neuronal tissue appears to have a disproportionate level of gene expression compared to other tissue. If this is related to behavioural plasticity, a comparison of gene expression in neonates and adults should prove interesting.

Gene expression in the developing brain has, unfortunately, been sadly neglected in arguments for gene expression in learning. One excellent study that pertains to this is the work of Chaudhari and Hahn (1983) who demonstrated that the adult mouse brain contains complex populations of polyadenylated (poly(A)+) and nonpolyadenylated (poly(A)-) mRNA's. These mRNA's were found to be separate sequence populations but, similar in complexity and in combination, equivalent to approximately 150,00 different mRNA sequences of average length. They report that, essentially, all the adult poly(A)+ mRNA are present in the brain at birth. In contrast, most of the

poly(A)- mRNA are absent. Brain poly(A)- mRNA's begin to appear soon after birth, but comparable levels to adulthood do not begin to appear until young adulthood. This could be compatible with both the work of Kandel in re-aggregated cells and research presented here in intact snails. That is, genes being expressed as a function of environmental exposure and learning. Such a notion linking environmental exposure and behaviour with gene expression is made of further speculative value by the recent evidence that poly(A)- mRNAs code for histone proteins, which are known to be involved in regulation of gene expression (see Lewin, 1987).

A gene expresses itself as follows: Ribonucleic acid transcribes the base sequence of that gene's part of the DNA chain to produce a complementary sequence. The RNA then leaves the cell nucleus and moves to the ribosome which translates the sequence into amino acid chains (proteins). Although all cells contain all genes, not all are expressed. Most of the genes are inactivated by histone proteins called 'repressors' which are coded by sites termed operators, which are next to the genes they control. When DNA replication occurs there is a necessary cessation of histone production. When replication is terminated, more histones are produced to hold the new DNA in check. Activation of genes appears to be accomplished by another protein type; nonhistone proteins. Unlike histones, which are fairly stable in composition and amount per cell, the nonhistones are, at least partly, in a state of dynamic flux. When the gene activity in a particular cell changes, one usually

detects a change in nonhistone proteins. Some of these are thought to switch on genes. The nonhistone proteins themselves are produced by genes. These feed back within the confines of a cell as a consequence of other chemical activity and are considered as "housekeeping" proteins. Of most interest here is that, nonhistones could come from other cells or their production could be triggered within the cell by other types of chemical signals. While histone proteins have generated considerable excitement in molecular neuroscience, nonhistone involvement have received little attention. If changes in gene expression do accompany learning, perhaps one future direction of research would be to detect nonhistone changes in neuronal tissue.

If new gene expression is self-maintained, which the data suggest by the duration of retention evidenced by the snails, two possible hypotheses emerge. First, specific histone production is switched off to maintain expression. Second, specific histones continue to be synthesised but are continually removed from a gene site.

Research in Helix suggests that long term habituation of the order of hours requires gene expression, and further training gives rise to stronger retention which becomes semi-permanent and, presumably, is the product of self-maintained gene expression. In light of the results presented here (in particular, Chapter 17), it could be hypothesised that secondary messengers responsible for post-translational modifications of existing proteins in short term habituation may phosphorylate histone proteins as well as membrane proteins thus initiate an initial gene

activity as suggested by Nagamine and Reich (1985). This gene activity could be expression of a functional protein or an operator protein. In that this gene expression can transcend subsequent protein synthesis inhibition, it is more likely to be a control or operator molecule.

Whichever mechanism proves to serve long term habituation, it must be maintained, thus, either a repressor that has been removed, must be continually be removed, if the repressor is continually synthesised, or the synthesis of the repressor must be permanently shut off. Both would require the action of more than one gene. Thus, it could be hypothesised that long term modifications of neuronal functioning such as long term habituation may require interaction with major control genes.

Major control genes have recently been indentified in developmental biology and are termed Homeo boxes. When activated by either histone removal or nonhistone sythesis, they have the power to alter multiple gene systems by switching on or off various gene systems subservient to them (e.g. Rubin, Tith, Patel, D'Eustachio and Nguyen-Huu, 1986). Recent advances in our understanding of homeo boxes stems largely from evidence on the control genes which determine the body plan of Drosophila. Gehring (1987) reported that Homeotic genes share a characteristic DNA segment, (the homeo box) which encodes a defined domain of the homeotic proteins. The homeo domain appears to mediate binding to specific DNA sequences whereby the homeotic proteins exert a gene regulatory function. Homeo box genes have now been identified in a variety of organisms and have advanced our

understanding of developmental plastic changes. Evidence of protein synthesis involvement in long term habituation presented here increases the relevance of the many recent advances in molecular embryology, and, of particular interest in light of results of Chapter 17, the concept of control genes or homeo boxes may greatly increase our understanding of multiple gene involvement in neuronal plasticity.

Initial gene activity from long term habituation may be more permanent than predicted by Kandel et al. and may culminate in long term alterations of multiple genes with perhaps changes in homeobox or major control systems. The demonstration and elucidation of parameters of PSI effects are of critical importance to our further understanding of these and other speculations. Having established PSI effects, the area of long term mechanisms is now open to attack from the rapidly expanding armoury of molecular biology such as reverse transcription, RNA/DNA hybridisation, immunobiology, and gene effect assay systems such as cell free systems or egg cell expression of exogenous genes.

Although, post-translational changes in proteins and gene expression are discussed separately, this does not infer that such processes are mutually exclusive, post-translational modifications of proteins may be the active process which learning induced gene expression codes for. Thus an investigation of any interaction of modified proteins and activity-dependent gene expression would advance our understanding, at the biochemical level, of the multiple processes.

Having presented evidence suggesting that new gene expression is required for long term habituation, and suggested possible mechanisms of how such a process could occur, it is worth intimating how the products of such a maintained gene expression may alter the functional capacity of neuronal systems.

### 18.5.3 GENE EXPRESSION AND FUNCTIONAL CHANGES IN PHYSIOLOGY

One possible way to test the function of newly expressed mRNA and other macromolecules is to examine their translation in a model system. One much utilised system is the frog oocyte. Sumikawa, Parker, and Miledi (1984) report that mRNA extracted from adult rat brains could be injected into frog oocytes where the RNA was translated. The products of these exogenous molecules were processed and incorporated into the oocyte membrane where they formed functional neurotransmitter receptors and voltage-operated channels. Different fractions of mRNA induced the incorporation of different transmitter receptors and voltage-operated channels. These experiments provide a useful step towards the understanding of the structure and function of neurotransmitter receptors and channels. The application of this technique to specific long term learning macromolecules could at last provide a tenable link between the genome and other biochemistries reported to be affected in learning at the membrane.

Numerous suggestions appear in the literature of how various biochemical changes may mediate functional changes

in intercellular communication that would be required in learning. Linking activity in the genome to these changes is now of critical importance. It must be borne in mind that however appealing a particular theory may be, it is imperative that the particular changes, be they in membrane proteins, glycoproteins other macromolecules, or bistable switches, they must be replicable beyond half lives of constitutive molecules (see Chapters 4 and 17).

#### 18.5 SUMMARY AND CONCLUSIONS

This thesis provides evidence that the snail Helix aspersa can show both short and long term habituation of the dorsal antennae withdrawal response, and that such long term habituation is far from a transient phenomenon. Further, these short and long term processes show parametric characteristics demonstrated and extensively researched in vertebrates. The requirement of protein synthesis in long term learning in an intact animal was demonstrated, and PSI induced amnesia was further investigated. It has been argued that maintained alterations in gene expression are required to perpetuate initial protein synthesis dependent retention. The involvement of protein synthesis in long term habituation but not in short points to mediation of these phenomena by different processes. Further, the evidence presented here appears to point to a three stage process of short and long term habituation.



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## APPENDIX 7.1

A series of leucine  $^3\text{H}$  incorporation studies were conducted by colleagues in support of this thesis following the methodology and procedure outlined by Schwartz et al., (1971). As this work does not constitute part of this thesis, the brief description supplied with the results is included as an appendix and is discussed in light of this fact.

### STANDARD INCUBATION CONDITIONS FOR AMINO ACID INCORPORATION

Test snails received ganglionic injections of 20  $\mu\text{l}$  leucine  $^3\text{H}$  (specific activity 30-40mc/mole) in 50  $\mu\text{l}$  of physiological saline containing 50 mM Tris-HCl buffer (pH7.7), streptomycin (0.1mg/ml) and penicillin G (200U/ml) following Schwartz et al., (1971).

### PREPARATION OF TISSUE FOR SCINTILLATION COUNTING

The ganglia were dissected from the snail after the incubation period ascribed in Chapter 7. The ganglia were immediately frozen in liquid nitrogen and powdered in a small glass homogenizer. After precipitation with 5% Trichloroacetic acid (TCA), the protein was kept at 90 C for 20 mins, and subsequently washed on glass-fibre pads with 5% TCA, and, ethanol:ether (1:2), then dried with

ether. The pads were counted by scintillation in a toluene based scintillation fluid.

#### THE EFFECTS OF ANTIBIOTICS ON INCORPORATION INTO PROTEIN

The time course of leucine incorporation into ganglia proteins in the presence of each antibiotic at each dose was investigated and is reported in Chapter 7. These ganglia were incubated under standard conditions (400 counts/min per pmol) as described above in this appendix. At the incubation intervals indicated in Chapter 7, protein from the ganglia were precipitated and prepared for scintillation counting as described above, and the amount of leucine incorporated into protein is reported (pmoles/ganglion) in Figures 7.1 to 7.4.

Conclusions from these studies however must remain tentative in light of the lack of supportive data.

These supplied results, and also those reported in Chapter 8 - 17, are particularly interesting in light of the apparent similarities of antibiotic dose effects despite their differences in molecular weights (Anisomycin, 265.30; Cycloheximide, 281.4; Puromycin, 471.5; & Actinomycin D, 1255.4). With respect to this variance in molecular weights, these results show a marked similarity between drugs in terms of their dose effect relationship. Interestingly this commonality of dose effect relationship was also found in the behavioural Chapters (8 - 17). Thus, it cannot be

discounted that these antibiotics have a behavioural effect by a common mechanism as well as, or instead of their own specific modes of action (see Chapter 1). One possibility which cannot be ruled out by these labelling studies is that the antibiotic effects on leucine incorporation could indicate that the drugs disrupt amino-acid transport. Robertson, (1978), reports that pharmacological disruption of amino acid transport immediately after training disrupts long term memory. Further investigations would be of interest in this area, as would similar studies using other radioactive amino acids.