EFFECTS OF CYCLOHEXIMIDE, A PROTEIN SYNTHESIS INHIBITOR, ON LEARNING AND RETENTION IN THE COCKROACH, PERIPLANETA AMERICANA

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ABSTRACT

EFFECTS OF CYCLOHEXIMIDE, A PROTEIN SYNTHESIS INHIBITOR, ON LEARNING AND RETENTION IN THE COCKROACH, PERIPLANETA AMERICANA

By

Kathryn Lee Lovell

Cycloheximide, a protein synthesis inhibitor, has been used to test the theory that memory requires ongoing synthesis of brain proteins. Research on vertebrates has demonstrated that cycloheximide, when administered before training, impairs the formation of long-term memory, but has no effect on acquisition. However, previous experiments on headless cockroaches trained to lift a leg to avoid shock indicated that cycloheximide impaired the rate of acquisition, but had no observed effect on retention. The experiments reported in this dissertation were initiated to investigate this apparent difference in the effects of cycloheximide on learning and memory processes in cockroaches and vertebrates, and to further characterize the effects of cycloheximide in cockroaches.

Experiments were designed to investigate whether the cycloheximide-induced acquisition impairment is a general phenomenon in cockroach learning or is specific to the leg lift paradigm in headless cockroaches. Cycloheximide was injected before training into cockroaches trained under one of three conditions: (1) headless cockroaches trained to lift a leg to avoid shock; (2) intact cockroaches trained to lift a leg to avoid shock; (3) intact cockroaches trained to turn left or right in a

T-maze to avoid shock. The results confirmed that cycloheximide prolongs the time required for headless cockroaches to reach a given criterion of leg lift learning and demonstrated that cycloheximide has no impairing effect on acquisition in intact cockroaches trained to lift a leg or in intact cockroaches trained in a T-maze. Thus, the cycloheximide impairment effect is specific to the headless preparation.

Experiments were performed to explore the nature of the cycloheximide-induced acquisition impairment in the headless preparation, i.e. whether it is an effect on the learning process which operates in the ganglia of headless cockroaches, or a peripheral effect on activity levels of the leg, making performance of the leg lift task more difficult. Analysis of the leg behavior of yoked controls was performed to determine if cycloheximide altered the leg activity levels (as measured by the number of flexions and extensions) during the leg lift training period. Yoked controls received the same amount of shock as experimental animals, but the receipt of shock was not correlated with leg extension. Cycloheximide had no observable effect on the leg activity of the yoked controls, which would support an effect of cycloheximide on learning. However, cycloheximide did significantly increase the leg activity of the experimental animals at the beginning of the training period, which would suggest an effect of cycloheximide on activity. Preliminary behavioral and electrophysiological experiments were done to investigate possible effects of cycloheximide on the peripheral nervous system. No gross effects of cycloheximide were observed (a) on the magnitude of shock necessary to produce twitching of the leg or (b) on evoked electrical activity of nerves 5 and 6 in response to

electrical or mechanical stimulation. Since they do not demonstrate an effect of cycloheximide on peripheral nerve activity, these experiments are consistent with an effect of cycloheximide on learning. In view of the conflicting results on the nature of the cycloheximide impairment effect, further experiments are necessary to resolve this question.

The effects of cycloheximide on retention were investigated in cockroaches trained to turn left or right in a T-maze to avoid shock. Cycloheximide was injected before training, and retention was tested at intervals up to 22 hours after training. A dose of cycloheximide which inhibited protein synthesis in the nervous system by over 90% did not produce retention deficits at any interval in this training situation, although it increased the activity of the animals in the maze. This is in marked contrast to the severe memory impairment observed in mice after injections of cycloheximide which produce similar degrees of protein synthesis inhibition. Additional experiments utilizing various training conditions are necessary (a) to determine if it is possible, under any conditions, to produce amnesia in cockroaches by administration of cycloheximide, and (b) to identify any fundamental differences which may exist in the memory consolidation processes of cockroaches and mice. Such comparative studies are important in examining the phylogenetic evolution of molecular mechanisms of learning and memory.

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Kathryn Lee Lovell

A DISSERTATION

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Dedication

This dissertation is dedicated to my husband, Bob,
who has given loving and patient moral support
throughout my academic career,
and to my daughter Jennifer.

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INTRODUCTION AND REVIEW OF LITERATURE

"Living matter and clarity are opposites--they run away from each other."

---Albert Einstein

The search for the underlying cellular and molecular mechanisms of learning and memory has yielded a great number of experimental results, but no simplifying conclusions have yet emerged. Inherent in such research is the difficulty of defining precisely what "learning" is. There are many types of behavioral modifications, including classical conditioning, instrumental conditioning, and habituation, which may not necessarily be mediated by identical physiological processes. The research reported here was conducted on the assumption that an understanding of the molecular and cellular bases of any interesting behavioral modification is likely to provide principles of neural plasticity which are of wide generality. However, it should be kept in mind that this dissertation includes two very different types of cockroach learning experiments.

One aspect of the search for underlying molecular mechanisms has involved an investigation of the role of protein synthesis in memory. The fundamental importance of proteins in regulation of cell functioning suggests the hypothesis that brain proteins may be involved in memory processes. Research investigating the validity of this hypothesis in vertebrates has implied that protein synthesis is necessary for normal operation of long-term memory processes. Experiments investigating this

hypothesis in invertebrates are relatively rare. The research reported in this dissertation was undertaken to test this hypothesis in cockroaches and to explore some possible differences between cockroaches and vertebrates concerned with effects of cycloheximide (a protein synthesis inhibitor) on learning and memory processes. Such comparative studies are important in examining the phylogenetic evolution of molecular mechanisms of learning and memory.

In this literature review, previous studies of learning in cockroaches will be discussed first to provide an orientation to the types of paradigms in which cockroaches have been trained. Next, characteristics of learning in insects will be compared to those of vertebrates to provide an evolutionary perspective of some "laws of learning". In the third section, experiments investigating the effects of protein synthesis inhibiting drugs on memory in vertebrates will be summarized. Finally, the few experiments exploring behavioral effects of cycloheximide (CXM) in cockroaches will be discussed.

Characteristics of Learning in Cockroaches

The characteristics of learning in cockroaches have been investigated in three general types of learning paradigms during the last 70 years. In two of these paradigms--dark avoidance learning and learning in various types of mazes--intact animals have been trained, while the third type of learning--leg position learning--has been demonstrated in the ventral nerve cord and in an isolated thoracic ganglion, as well as in the intact animal. These training situations will be described, conclusions about cockroach learning abilities summarized, and some biochemical and electrophysiological aspects of the learning

will be discussed. Other reviews of learning in cockroaches have been written by Guthrie and Tindall (1968), Alloway (1973), and Miller (1970).

Dark avoidance learning procedures, which comprised the first paradigm used in cockroach experiments, were devised independently by Szymanski (1912) and Turner (1912). In both cases, the apparatus consisted of a closed box with a lighted side and a darkened side. When a cockroach was placed in the lighted side of the box, it immediately entered the darkened side since cockroaches prefer dark to light. However, the floor of the darkened side was a grid through which shocks were administered, causing the animal to run back into the lighted side. After varying amounts of exposure to the shock, the animals learned to remain in the lighted area. Various versions of this dark avoidance training procedure have been used by Minami and Dallenbach (1946), Hunter (1932), and Lovell and Eisenstein (1973).

A number of maze situations have also been used for training cockroaches. Turner (1913) first used a flat, elevated maze mounted over water. An overhead light served as a negative reinforcer from which the animal could escape by entering its home cup, mounted at the goal end of the maze. This type of maze has been used by Gates and Allee (1933) and a number of French investigators (see Alloway, 1973). Chauvin (see Guthrie and Tindall, 1968) trained animals in a relatively complex maze--nine blind alleys--with a dark box as the goal. Eldering (see Guthrie and Tindall, 1968) used simpler mazes, including training cockroaches to turn either right or left to avoid an electric shock. Longo (1964) trained cockroaches in a simple Y-maze using electric shock as negative reinforcement. In these experiments, training was conducted over a period of days. Negative reinforcement,

employed in all of the above training situations, has been found to be the most reliable reinforcement in cockroach training.

The third type of training situation utilized for cockroaches is leg position learning. This procedure for studying learning in headless insects was first described by Horridge (1962) and later investigated by others (Eisenstein and Cohen, 1965; Disterhoft et al., 1971; Eisenstein, 1970a, 1970b, 1972b; Disterhoft, 1972). During the initial training period one animal of a pair, called the positional or P animal, received a shock whenever it lowered its leg lead into a saline bath, completing a circuit. A second animal, called the random or R animal, was connected in series with the P animal, so that both animals received shocks until the P animal lifted its leg out of the saline. Thus the R animal served as a yoked control, receiving shocks when its leg was in a variety of different positions while the P animal was only shocked when its leg was extended. Following a 45 minute training period, the circuit was altered for a testing period so that each animal received shock independently when its leg was extended. Since both animals received the same amount and pattern of shock during training, any difference in behavior could be attributed to the specific association between leg extension and shock experienced by the P animal. During training P animals learned very quickly to flex their legs to avoid shock--an asymptote was reached in about 1.5 minutes (Eisenstein, 1972) and thereafter very few shocks were initiated. In contrast, the R animals kept their legs extended into the saline solution a majority of the time throughout the training period. During the test period, P animals initiated significantly fewer shocks than the former R animals. During testing, acquisition of the leg flexion

response by R animals was inferior to naive P animals. This has been interpreted as indicating that the R animals learn that shock administration is not correlated with leg position and this learning interferes with acquisition of the leg flexion response (Eisenstein and Cohen, 1965), a theory which has been confirmed by Disterhoft et al. (1971).

Most of the leg lift experiments have been performed on headless animals, demonstrating that learning can occur in the ventral nerve cord. Intact animals have also been trained (Disterhoft et al., 1968; Pritchatt, 1968, 1970) although acquisition usually takes longer than for headless animals. In addition, Eisenstein and Cohen (1965) showed that leg lift learning could be mediated by an isolated ganglion. These experiments have been reviewed by Eisenstein (1972b). Retention of the leg flexion task has been demonstrated for up to 72 hours in headless animals (Harris,1971) and up to 48 hours in intact preparations (Disterhoft et al., 1968). Thus in these experiments headless cockroaches acquired the leg lift learning more quickly and retained it longer than intact cockroaches.

Chen, Aranda and Luco (1970) conducted some classical conditioning experiments on cockroaches and found that the head was necessary for learning, although not for retention after acquisition of the learned response. They trained intact cockroaches, headless cockroaches, and isolated ganglion preparations to avoid electric shocks by leg flexion, contingent upon a mild electric signal which was applied a fraction of a second prior to the shocks. All the intact animals learned to avoid the shocks in approximately 10 sessions on a schedule of two daily sessions and 10 trials per session. The headless

and isolated ganglion preparations failed to acquire the response on this schedule, although the headless animals learned if given massed training of 14 sessions per day, which would suggest a shorter retention span. Retention tests showed that there was no decrement of avoidance reactions in intact animals until the end of insect life (which occurred 3 to 8 days after training,due partly to suspension of the animals on a rod without access to food or water and partly to surgical damage). The retention was not affected by the severance of the head or by isolation of a single ganglion, once the behavior had been established. Thus in this training situation (proper controls have not been and cannot be run to establish it as true classical conditioning), the head ganglia are apparently necessary for learning to be most effectively established, but are not the only site of memory storage. There would appear to be communication of some type among ganglia of the nerve cord during training in order to simultaneously establish memory in the ventral nerve cord and the brain.

Transfer of information between ganglia during training of cockroaches in Horridge's leg lift procedure has also been observed. If a prothoracic leg is trained and a mesothoracic leg (Harris, 1971) or metathoracic leg (Horridge,1962) is tested, the latter legs on a P animal are superior to the corresponding legs on a naive or R animal. The transfer results of Harris have been confirmed (Reep and Eisenstein, unpublished).

Hoyle (1965) and Aranda and Luco (1969) have reported electrophysiological studies concerning leg flexion learning. Hoyle (1965) recorded the activity from single muscles that control leg flexion in the locust leg--usually the metathoracic coxal adductor, which is

supplied by a single excitatory motoneuron. Shock was delivered to the leg whenever the discharge frequency in this motoneuron fell below an arbitrary "demand" level. As a result the motoneuron was induced to maintain an elevated discharge level. When shocks not contingent on discharge frequency were given, there was no sustained increase in the discharge level of the motoneuron. The reported results are based on only two animals, but this study, if confirmed, provides a good example of an electrophysiological correlate to operant conditioning.

Aranda and Luco (1969) trained metathoracic ganglia of Blatta orientalis in the leg lift paradigm, dissected them out of the animals and maintained them in vitro. When Aranda and Luco recorded from nerve 5 emerging from the ganglion, they observed increased responsiveness to electrical stimulation of the ventral nerve cord in the trained side of ganglia from P animals, but no comparable increase in responsiveness from the untrained side of P ganglia or either side of R ganglia. Apparently neuronal or synaptic changes in the ganglia occurred as a result of leg extension and shock pairing, but not as a result of non-contingent shock administration alone, leading to increased nerve activity.

The effects of various pharmacological agents on leg lift learning, and biochemical correlates of the learning, have been studied in a few experiments. Details of the effects of CXM will be discussed in a later section. Kerkut, et al. (1970) reported changes in the rate of acquisition of the leg lift task after administration of a number of drugs to headless cockroaches. Actinomycin D, acridine orange, congo red, chloramphenicol, and CXM increased the time taken to achieve a given criterion, while edrophonium, prostigmine, physostigmine,

amphetamine and magnesium pemolate decreased the time to criterion. The time taken to reach a given criterion, or an asymptotic level, could be altered either by a change in the rate of learning (i.e., the slope of the learning curve) or by a change in the initial activity levels of the animals. From the data presented in the paper by Kerkut, et al. (1970), it is not possible to distinguish the effect of the drugs on the rate of learning from their effect on initial activity levels, and the mode of action of the drugs needs to be clarified. Hereafter the term "change in rate of acquisition" or "impairment of acquisition" will be used to refer to changes in the time taken to reach criterion, caused either by changes in activity or in the learning process. The term "learning impairment" will be reserved for cases where it can be demonstrated that activity effects are not responsible for acquisition changes produced by a drug.

After training the cockroach metathoracic leg in shock avoidance, Kerkut, et al. (1970, 1972) found that the metathoracic ganglia of P animals showed increased RNA turnover and protein synthesis as compared to R ganglia and untrained controls. The changes in the P ganglia were localized in the posterior half of the ganglia, where the cell bodies of motoneurons innervating flexor and extensor muscles are located. Sukumar (1975) also reported an increase in RNA concentration in the metathoracic ganglia of grasshoppers trained in the leg lift procedure as compared to the ganglia of yoked control and resting animals. Cholinesterase activity and production of GABA (gamma-amino butyric acid) were lower in the ganglia of P animals after training (Kerkut et al., 1970). The recovery of cholinesterase activity closely paralleled forgetting of the learned response over a three day period.

Acetylcholine and GABA have been identified as probable synaptic transmitters (excitatory and inhibitory, respectively) in the central nervous system of the cockroach (see Gerschenfeld, 1973), implicating synaptic transmission systems in learning. However, two other studies (Woodson, et al., 1972; Willner and Mellanby, 1974) failed to detect any change in cholinesterase activity in the cockroach ganglion as a result of leg lift training (see Davis, 1975, for further discussion).

In research investigating the role of the cyclic AMP system in learning, Nathanson (1973) administered several drugs to headless cockroaches trained to lift or extend a leg to avoid shock. Drugs which potentiate the action of cyclic AMP by preventing its metabolic breakdown (theophylline and SQ 20006) and dibutyryl cAMP (which has the same cellular effects as cyclic AMP) significantly reduced the average time taken during acquisition to reach a given criterion. Similar drugs which have no effect on cyclic AMP concentrations, including 5'-AMP (the biologically inactive product of cyclic AMP breakdown) and sodium butyrate, had no effect on the rate of acquisition. Since cyclic AMP has been proposed as a mediator of the postsynaptic action of transmitters (see Nathanson,1973), these studies suggest the importance of synaptic events in learning.

Comparison of Some Aspects of Learning in Insects and Vertebrates

Although behavioral studies of learning in insects are scarce compared to those of mammals, enough data has been obtained to observe some similarities and some differences between learning characteristics of the two groups. The similarities will be discussed first.

The interference theory of Jenkins and Dallenbach (1924) which postulates that forgetting is mainly due to interpolated activity has been confirmed on cockroaches as well as vertebrates. Minami and Dallenbach (1946) compared the effects of forced activity and of inactivity on relearning. Forced activity after training decreased retention of a dark avoidance response in cockroaches while inactivity between training and testing, produced by wedging subjects between folds of tissue paper, greatly increased retention compared to controls given corresponding intervals of normal rest.

Kamin (1957) first observed that retention of dark avoidance learning in rats decreased to a minimum at 1 hour after training and subsequently increased over a period of 19 days. A similar pattern of retention, with a minimum at 1 hour, has been seen by Eisenstein (1970a) in an investigation of shock avoidance learning in the headless cockroach. Also, a "Kamin effect" with a retention minimum at 2 days was observed by Alloway (1969) in the grain beetle, using a number of spaced training trials in a maze. The Kamin effect has been interpreted as indicating a transition between two stages of memory, such that at the lowest point of retention, short term memory has decayed and long term memory has not yet been fully established. Thus the existence of the Kamin effect in insects suggests the occurrence of a two stage memory process involving a short term phase before the memory becomes permanently established in long term storage, as has been widely proposed for vertebrates.

Further evidence for a two stage hypothesis can be obtained from retrograde amnesia studies, which are common in vertebrates. Generally, in such experiments, retrograde amnesia is observed if one of many

physical or chemical agents is administered immediately after training. Retrograde amnesia is not observed if the agent is administered some period of time (which may vary from seconds to hours) after training. Results have been interpreted as suggesting "consolidation" of the memory trace from a short term stage to a long term stage such that disruption of memory may occur during the unstable short term stage, but not during the permanent long term stage. Several examples of retrograde amnesia have been observed in cockroaches. Minami and Dallenbach (1946) reported that treadmill running interfered with retention in the cockroach only when it was administered immediately after training. Treadmill running administered an hour after training had no effect on retention. The administration of carbon dioxide (an anesthetic which causes convulsions before quiescence) immediately after each daily learning trial prevented passive avoidance learning in cockroaches (Freckleton and Wahlsten, 1968). Lovell and Eisenstein (1973) administered carbon dioxide (CO_2) to cockroaches immediately after or 1 hour after dark avoidance training, and measured retention 2 hours after training. When CO_2 was given immediately after training, no retention was observed 2 hours later. When CO_2 was given 1 hour after training some retention was observed. All of these experiments indicate that memory stages which differ in their susceptibility to disruption can be observed in cockroaches. Thus the nature of the memory trace changes with time in insects as it does in vertebrates, although whether the physiological substrates of the change are similar remains to be determined.

Similarities in characteristics of learning and memory were discussed above but there are also differences in learning between insects and vertebrates. For example, Longo (1964) studied probability learning in cockroaches. He used a T-maze in which the two arms were both reinforced, but with different probabilities. It was found that the cockroaches matched their probability of choice of an alternative to the probability of its being reinforced; for example, an arm that was reinforced 70% of the time was chosen 70% of the time. Thus the cockroach behavior differs from that of mammals, who maximize (choose the more frequently rewarded alternative about 100% of the time), and resembles that of fish and pigeons, who also match probabilities (Bitterman, 1965).

Another difference between cockroaches and rats can be seen in the form of the learning curves for animals trained in dark avoidance or maze learning. The insect curves exhibit a backsliding tendency in the early part of training before the final learning criterion is reached. This tendency is not seen in higher animals such as rats. This observation suggests the interpolation of a phase of instability in the course of some types of training, which then gives way to a more steady response in most insects, as might occur if the supply of some substrate had been temporarily reduced (Guthrie and Tindall, 1968).

In a learning reversal experiment conducted by Longo (1964), cockroaches were trained to turn either right or left in a T-maze and then the correct side was repeatedly reversed. No evidence was found to indicate that experience in learning reversals led to any improvement in the animals' ability to learn reversals. Conflicting results have been obtained in experiments on reversal learning in other insects

(ants: Schneirla, 1962; sow bugs: Thompson, 1957; McDaniel, 1969), so the ability of insects to demonstrate reversal learning is not clear. Progressive improvement in habit reversal has been easily demonstrated in the rat and pigeon but has been difficult to find in the fish (Longo, 1964).

An inability of cockroaches to show reversal learning would seem to be an example supporting a high degree of situation specificity in cockroach learning. Further support for this theory is provided by Chauvin (see Guthrie and Tindall, 1968), who concluded on the basis of a number of maze studies, that learning in the white rat is guided by "general methods" transferable to different problems, while in the cockroach the response varies with the type of question asked. Whether the structure or complexity of the insect nervous system precludes more complex learning has not been determined. It is possible that in the animals tested the type of learning demonstrated is that which is the most evolutionarily advantageous, and perhaps the tasks chosen were not those which would show the greatest potential of the animal for relatively complex learning.

Effects of Protein Synthesis Inhibitors in Vertebrates

The hypothesis that brain protein synthesis is an essential physiological process in the establishment of long term memory has been widely proposed. Since proteins serve a number of functions in cells, they could possibly alter interneuronal connections and thus serve as the basis of behavioral changes. Barondes (1965) has proposed several ways by which biosynthesis of proteins could alter intercellular relationships. For example, newly synthesized proteins which

have been transported to pre- or post-synaptic neuronal sites might alter (a) the proximity of neural connections, (b) membrane permeability, (c) rate of synthesis or degradation of a transmitter, or (d) the number of functional interneuronal contacts. Many experiments have been conducted on the assumption that a drastic reduction in protein synthesis will impair memory if, in fact, memory processes require the synthesis of new or altered brain proteins. Reduction in protein synthesis by more than 90% can be achieved by administering one of several antibiotics: puromycin, cycloheximide, acetoxycycloheximide, or anisomycin.

Generally in these experiments an animal is given an antibiotic shortly before or after the acquisition of a learned response. At some time after training the animal is tested for retention of the response. These experiments have been interpreted according to the postulate: if it can be shown that a drug-treated group is impaired in retention as compared to a control group, and this impairment cannot be attributed to a nonspecific effect of the drug, then the impaired retention may be taken as evidence that protein synthesis is required for some aspect of the memory process. However, all of the drugs used have physiological effects other than inhibition of protein synthesis which may cause behavioral changes. Differing effects of the several antibiotics on behavior under constant conditions of protein synthesis inhibition have been noted, and recovery of memory has occurred under certain conditions. Such complications have led to some difficulties in the interpretation of the role of protein synthesis in memory formation.

Selected experiments in this area will be discussed below. The discussion will be organized according to the type of antibiotic used. It should be remembered that a great many variables are involved in this type of research. Experiments differ as to dose level, the location of injections, the species of animal, the type of learning task and extent of training, and the temporal relationships among training, drug injection, and retention test, and results do not always agree. Recent reviews in this area include articles by Barraco and Stettner (1975), Uphouse, et al. (1974) and Gibbs and Mark (1973).

Puromycin. Puromycin is an antibiotic which blocks protein synthesis by functioning as an analogue of amino-acylated tRNA. Specifically, it substitutes for an incoming amino-acyl tRNA as the acceptor of the carboxyl-activated peptide, causing premature release of polypeptide chains of various lengths from ribosomes (Nathans, 1964). This drug is very effective in producing amnesia when injected intracerebrally either before or after training.

Puromycin was first used in memory impairment studies by Flexner, et al. (1962,1963). In their studies, mice given bilateral injections of puromycin (producing protein synthesis inhibition of 80-90%) in the temporal lobes 1-3 days following acquisition training showed a loss of memory when retrained a few days after the injections. Learning was not affected since animals receiving puromycin treatment were able to relearn the maze and were capable of reversal learning. The disruption of memory by injections of puromycin given more than a few hours after training (delayed injections) reportedly could be

reversed with saline injections (Flexner et al., 1963) and thus did not appear to be a permanent amnesia. When puromycin was injected immediately before or immediately after training, the amnesia produced appeared to be permanent and could not be reversed with saline injections. These experiments indicate that puromycin may inhibit memory by two different mechanisms--1) it may temporarily prevent retrieval of stored information in the delayed injection experiments, while 2) it may inhibit consolidation of the memory trace when injected close to the training period and thus induce a permanent loss of memory. However, not enough experiments have been done to confirm the permanency of amnesia produced by injections before training.

Barondes and Cohen (1966) found that when puromycin was injected 1-5 hours before training, acquisition was normal but in the 2-3 hours after training, the drug treated animals showed a progressive decline of retention. In goldfish (for example, see Agranoff et al., 1966) puromycin administered before or immediately after training caused a retention deficit 1-3 days later. Delayed injections of puromycin, given an hour or more after training, had no effect on retention, unlike the results in mice.

Puromycin has many other physiological effects in the brain other than protein synthesis inhibition, and these side effects cannot be excluded as the cause of puromycin-induced memory impairment. This antibiotic has been shown to produce seizures shortly after intracerebral administration (Cohen and Barondes, 1967). Flexner and Flexner (1968) found evidence for the survival of peptidyl-puromycin fragments (formed by the incorporation of puromycin into a growing polypeptide chain which is prematurely released) in the brain for at

least several weeks after intracerebral injections of tritiated puromycin. Other side effects include mitochondrial abnormalities, inhibition of respiration in cerebral cortex slices, inhibition of 3'5' adenosine monophosphate phosphodiesterase (see Squire and Barondes, 1972), inhibition of rat brain acetylcholinesterase (Moss et al., 1974), and reduction of the amplitude of action potentials in the fish spinal cord by 25% (Bondeson, et al., 1967).

Glutarimides. Cycloheximide (CXM) and acetoxycycloheximide (AXM) are glutarimide derivatives. This class of antibiotics interferes with several steps involved in the translocation of the peptide chain along the ribosomes, including release of transfer RNA and movement of messenger RNA along the ribosome (Flood, et al., 1973). Inhibition occurs very quickly; for example, 15 minutes after subcutaneous injection of AXM, cerebral protein synthesis was inhibited by over 90%. Both intracerebral and subcutaneous injections of glutarimides have been used with similar effects on memory. Generally in the studies on mice and goldfish using CXM or AXM (see Barraco and Stettner (1975) for further details), animals showed normal learning when trained after injection, remembered normally 3 hours after training, but were amnesic by 6 hours and thereafter. Injections immediately after training had a less marked, but significant, amnesic effect. Injections made 30 minutes or more after training had no amnesic effect. Retention deficits were not observed under any condition if protein synthesis was inhibited by less than 80%. Thus, normal operation of long term memory processes seems to require synthesis of new proteins but, since amnesia did not become evident for several hours

after training, some type of short term memory process not dependent on protein synthesis must be operative for at least 3 hours after training.

Daniels (1971) investigated the effect of AXM by injecting AXM into the hippocampi of unrestrained rats through two cannulas. Cerebral protein synthesis was inhibited by 95% during training in a Y-maze, but acquisition was not affected. When injections were given 5 hours before training, memory was severely impaired at 6 hours, 24 hours and 7 days, but not at 3 hours after acquisition. When injections were given 5 hours before recall tests or immediately after acquisition, memory was unimpaired. This and other research using glutarimides suggest that CXM and AXM produce amnesia as a result of interference with: a) a system necessary for the storage of long term memory activity which is initiated at the time of acquisition; or b) a system necessary for the recall of long term memory which is established during acquisition. CXM and AXM do not appear to affect the initial acquisition process or the information retrieval process.

Effects of CXM on the later stages of acquisition have been demonstrated by Squire and Barondes (1973). When mice were given prolonged discrimination training to escape shock shortly after subcutaneous administration of CXM, acquisition was normal during the initial 15-21 trials. Beyond this point, mice given CXM did not continue to improve as rapidly as mice given saline. Squire and Barondes concluded from this and additional studies that the acquisition deficit is early evidence of progressive impairment in long term memory which is dependent on cerebral protein synthesis. They proposed that

a long term memory process, which is dependent on protein synthesis, has not only been established within minutes after the beginning of training, but is also required within minutes after the beginning of training for progressive improvement of performance. During prolonged training in certain situations, continued normal improvement of performance may depend on the establishment of memory of an increasing number of previous trials.

The occurrence and degree of amnesia produced by CXM or AXM are not always consistent among various experiments. A number of parameters have been identified which lead to differences observed in retention (Flood, et al., 1972; Quinton, 1974; Preache, 1973). The degree of training is one of the most important of these variables. In one-trial passive avoidance learning experiments reported by Flood, et al. (1972), the degree of amnesia produced by CXM varied inversely with the level of training. Several experiments have demonstrated that over-training animals may completely prevent the appearance of amnesia in CXM or AXM treated mice. However, there may be additional unidentified variables which also affect amnesia production.

There have been conflicting results reported on the permanence of the glutarimide induced amnesia. These considerations are important in trying to distinguish between interference with the storage of the memory trace as opposed to impairment of the retrieval process. Interference with storage mechanisms should produce a permanent amnesia; recovery from amnesia implies a deficit in retrieval and indicates that the storage mechanism must be intact. A few investigators have reported spontaneous recovery of memory in experiments where CXM was injected before training and memory impairment had been found

to be present 24 hours after training. Memory was restored at time intervals ranging from 58 hours to 14 days (see Barraco and Stettner, 1975). Thus the protein synthesis inhibition produced during training by glutarimides does not necessarily block the memory storage process. In addition, there are physiological and behavioral treatments which, when administered before testing, can produce good retention in animals at times when subjects not given the treatments show a high degree of amnesia. For example, repeated exposure to the training apparatus, or a "reminder shock" given under certain conditions, can trigger the recovery of memory after CXM-induced amnesia (Quartermain, et al., 1972). Thus some aspect of the memory trace appears to survive CXM treatment.

Barondes and Cohen (1968b) investigated the susceptibility of CXM induced amnesia to drugs involved in "arousal" mechanisms. It has been proposed that such drugs act by altering the adrenergic system, which presumably plays an important role in mediating arousal (Kety, 1970). When CXM-treated mice were injected with amphetamine 3 hours after training (before amnesia was observed) there occurred an "induction" of long term memory, such that retention was normal at 6 hours and 7 days after training. Similar findings have been reported using metaraminol, an adrenergic stimulant (Serota, et al., 1972). In line with these pharmacological experiments, Flexner, et al. (1973) demonstrated that CXM and AXM inhibit the activity of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of norepinephrine (NE), and suggested the possibility that the amnesic effect of the glutarimides may be due in part to reduction of the functional pool of NE.

Serota, Roberts and Flexner (1972) proposed the following model to explain the CXM effects on retention: (a) release of NE during training results in facilitatory changes in the task-specific network of synapses resulting in an "aroused" state; (b) during testing, release of NE results in specific reactivation of the previously facilitated network; (c) injections of AXM or CXM result in a decrease of NE with a correlative decrease in the degree of facilitation; (d) when animals are tested at 24 hours, levels of NE are still diminished,and consequently, the network is not reactivated and memory is not expressed; (e) finally, at 6 days (when memory had recovered in the experiments of these researchers) the noradrenergic system has recovered and memory is expressed. According to this model, administration of adrenergic agents (e.g. amphetamine, metaraminol) or application of certain behavioral manipulations (e.g. "reminder shock") at appropriate times will reactivate the partly facilitated network resulting in the expression of memory. Other experiments have provided support for this type of model (Randt et al., 1973; Botwinick and Quartermain, 1974). However, Squire et al. (1974) showed that alpha-methyl-p-tyrosine, a competitive inhibitor of tyrosine hydroxylase, in doses which depressed tyrosine hydroxylase activity as much as, or more than, CXM, did not affect memory. Therefore the effect of glutarimides on brain tyrosine hydroxylase activity is not sufficient to explain their amnesic effect.

Glutarimides have several physiological effects other than protein synthesis inhibition which could produce behavioral changes.

(a) Large doses may cause illness in animals, characterized by lethargy, diarrhea, or death. However, since animals can perform normally during acquisition under the influence of the drug, retention deficits cannot be attributed mainly to illness. (b) CXM, when injected subcutaneously or intracerebrally, produces large changes in the activity levels of mice. The drug first increases activity, then after about an hour, the activity level falls below control levels (Squire et al., 1970; Schneider and Chenoweth, 1971). However, activity effects can be dissociated from effects of CXM on memory (Segal et al., 1971). (c) Booth and Simson (1973) demonstrated that CXM can produce aversion to an odor if the drug is administered after ingestion of odorized food, suggesting that a part of the amnesia produced by CXM in discrimination tasks might reflect conditioned aversion to the cues present while the animal feels sick. However, Squire, et al. (1975) showed that lithium chloride produced as much conditioned aversion as an amnesic dose of anisomycin and more aversion than CXM, but did not affect memory. Therefore the aversive effect of protein synthesis inhibitors is not sufficient to explain their amnesic effect. (d) CXM also produces a prolonged alteration of established behavior patterns of nest-building in mice (Schneider and Chenoweth, 1971). The disruption of nest building continued for 3-5 days while activity levels and levels of protein synthesis returned to normal within 24 hours. Thus CXM may have prolonged effects on behavior which are not directly correlated with levels of protein synthesis inhibition.

On the cellular level, CXM disrupts brain polyribosomes, an effect which is independent of its inhibition of protein synthesis,

whereas protein synthesis inhibition and disruption of polysomes are closely linked in the case of puromycin (MacInnes and Luttges, 1973). The polyribosome disruption effect of CXM is specific for brain tissue, since this drug does not produce a massive disaggregation of polyribosomes from liver. Since it would appear that only excitable tissue such as brain tissue shows CXM-induced polysomal disruption it is possible that CXM effects might be linked to ionic mechanisms. This is supported by observations of alterations in the blood-brain barrier produced by CXM (MacInnes and Luttges, 1973). Further, this possibility is suggested by the similarity of effects of CXM, Ca^{++}, Mg^{++}, KCl, putative transmitters, ouabain, electroconvulsive shock, and inhibitors of oxidative phosphorylation on synaptosomal protein synthesis (see Barraco and Stettner, 1975). All of these agents inhibit synaptosomal protein synthesis. It has been proposed that a transport mechanism, requiring sodium and potassium for activation, exists and that many of the observed effects of the above agents are due to competition for transport into synapses or a transient disturbance of ionic balance for sodium and potassium which are necessary for maximum activity of synaptosomal protein synthetic systems.

Thus, CXM, as well as puromycin, has diverse and complex effects on cellular metabolism and, in particular, on neuronal metabolism, and it is important that care be exercised in interpreting correlations between behavioral and chemical events.

Anisomycin. Anisomycin (2-p-methoxyphenyl-3-acetoxy-4-hydroxypyrrolidine) is an effective, nontoxic inhibitor of cerebral protein synthesis which has recently been used in behavioral experiments

(Flood et al., 1973, 1974, 1975; Squire and Barondes, 1974; Squire and Davis, 1975). The duration of inhibition produced by this antibiotic is about 2 hours, much shorter than that of CXM. Since anisomycin (Ani) is relatively non-toxic, successive injections can be given, permitting variation of the duration of inhibition from 2 to 10 hours. Ani and CXM produce similar levels of protein synthesis inhibition and have similar amnesic effects, but Ani has opposite effects on locomotor activity than CXM--immediately after Ani injection, mice exhibit a slight depression of activity, in contrast to the hyperactivity shown by mice immediately after CXM injection (Squire and Barondes, 1974).

In contrast to the mechanism of inhibition of CXM, Ani does not appear to interfere with either peptide chain initiation or translocation, but instead interferes with the transfer reaction subsequent to the formation of amino-acyl transfer RNA, either by interfering with the catalytic center or by interaction with the peptidyl transferase (Flood et al., 1973; Grollman, 1967). Experiments with Ani are important in determining the influence of duration of protein synthesis inhibition on production of amnesia and in providing a third mechanism of inhibition with which to compare behavioral effects.

In several experiments, Ani produced amnesia in mice given passive avoidance training with characteristics similar to CXM-induced amnesia. Successive injections of Ani were used to vary the duration of inhibition (Flood et al., 1973, 1975). For each increase in training strength that blocks amnesia, there is a longer duration of inhibition that will reestablish the amnesia. There is a significant correlation between amount of cerebral protein synthesis inhibition, length of protein synthesis inhibition and the degree of memory impairment.

Squire and Barondes (1974) compared the effects of CXM and Ani on mice given discrimination training. Like CXM, Ani had no effect on acquisition with brief training, but impaired performance of mice during prolonged training. The similarity of the effects of Ani and CXM on memory impairment supports the hypothesis that the synthesis of new protein is required for a long term memory process which is initiated during training.

Summary. Puromycin injected from 5 hours before training to 24 hours or more after training causes a memory deficit which varies in its degree of permanence depending on the conditions. In contrast, CXM or AXM given after learning usually fails to produce retention loss, although injections within a few hours before training do reliably produce retention deficits. As in the puromycin experiments, the degree of training is an important variable in controlling the amount of memory loss. Amnesic effects due to all antibiotics tested develop over a period of several hours. Experiments with Ani showed that there is a correlation between amount of protein synthesis inhibition, length of the inhibition period, and amount of retention deficit. Thus, it appears that some aspect of the long term memory process requires synthesis of new protein for normal operation.

However, amnesia production is not a simple result of protein synthesis inhibition. Different drugs producing similar degrees of inhibition may have differing effects on memory. The differential effects produced by individual antibiotics may be due to (Barraco and Stettner, 1975): (1) the particular mechanisms involved in inhibiting protein synthesis and products of this specific inhibition

(e.g. peptidyl-puromycin fragments); (2) metabolic effects apparently independent of protein synthesis (e.g. puromycin appears to inhibit cyclic AMP phosphodiesterase independently of its inhibition of protein synthesis); (3) purely structural or stereochemical mechanisms, e.g. by acting on membranes to produce changes in permeability to ions or metabolites, or by acting on receptors to change neurotransmitter binding characteristics. In addition, dose level may be an important variable in determining which of the above effects are produced. Thus, the complex metabolic effects of these drugs complicate the interpretation of behavioral experiments.

Effects of Cycloheximide on Cockroach Behavior

A few experiments have been performed investigating the effects of CXM on leg lift learning in cockroaches (Kerkut et al., 1970; Brown and Noble, 1967, 1968; Glassman et al., 1970). Kerkut et al. (1970) and Brown and Noble (1967) found that an injection of CXM an hour before the beginning of training produced inhibition of the rate of acquisition in P animals (see page 4 for a description of leg lift learning). That is, the drug treated cockroaches reached the same criterion as the control animals, but the drug treated animals required a longer period to reach that criterion. A dose response curve was obtained in both studies, such that animals given larger doses of CXM required a longer time to reach criterion. It is impossible to compare doses and behavioral effects between these two experiments since the two studies used different legs (which showed different rates of learning) and different methods of injection in which widely varying doses of CXM were administered. Brown and Noble used doses of

2.5 µg to 12.5 µg of CXM applied to the ventral surface of the prothoracic ganglion and trained the prothoracic leg. Kerkut et al. injected doses of 50 µg to 150 µg into the haemocoele of the animal and trained the metathoracic leg. The highest dose used by Brown and Noble (1968) resulted in inhibition of ^{3}H-lysine incorporation of more 90%, although an inhibitory effect on the rate of acquisition was achieved at doses causing inhibition of 50% to 90%. There was approximately 90% inhibition of protein synthesis at the dose levels used in the research of Kerkut et al. (personal communication, G. W. O. Oliver). Both of these experiments were interpreted as supporting a CXM-produced impairment of learning.

However, Eisenstein (1968) suggested that CXM could be impairing performance rather than actual learning, since proper controls were not reported to rule out the possibility that CXM impairs the rate of acquisition by altering the leg activity or sensitivity of the leg to shock. Glassman et al. (1970) investigated this possibility by training headless animals for 10 minutes, injecting them with either CXM or saline, and resuming training after 5 minutes. The performance of the CXM-injected animals was impaired during the retraining period; i.e. they received significantly more shocks than control animals. Since both groups had previously reached criterion, the effect of CXM may not be on primary acquisition, but on performance of the leg lift task. It is also possible that CXM caused amnesia, although testing was conducted 5 minutes after training and the retrograde amnesia seen in vertebrates usually is not observed for several hours after training.

Brown and Noble (1967) observed an impairing effect of CXM on retraining, as well as on initial acquisition, in cockroaches in which the prothoracic ganglion was isolated and the prothoracic leg trained. During initial acquisition, drug treated animals required a mean of 19.6 minutes to achieve criterion versus a mean of 3.9 minutes for controls. During retraining 60 minutes after the end of training, CXM treated animals required a mean of 6.3 minutes versus 1.8 minutes for controls. Although these results could be interpreted in terms of effects of CXM on learning and memory, they are consistent also with an effect of CXM on performance of the leg lift task.

Kerkut et al. (1970) also looked at the effects of CXM on retention. When animals were injected 15 minutes before training and tested 6-12 hours later, there was no significant difference between the retention of CXM-injected and saline-injected animals (personal communication, G. W. O. Oliver). Also, CXM injected after learning had taken place had no effect on retention (Kerkut et al., 1970). Thus, CXM does not appear to produce amnesia in cockroaches trained in the leg lift paradigm.

The behavioral effects of CXM have been investigated in only one type of invertebrate--cockroaches--and in only one type of paradigm--headless animals trained in leg flexion. This paradigm has the advantage of reducing the amount of nervous tissue involved in learning, with the goal of eventually identifying the neurons participating in the behavioral changes. However, results obtained with this paradigm are very sensitive to changes in leg activity. Also, the reliability of considering headless or isolated ganglion

preparations as model systems for studying learning as it occurs in the intact animal has been widely assumed, but the question has never been explicitly investigated.

RATIONALE OF EXPERIMENTS

The effects on learning and retention of pharmacological agents which inhibit protein synthesis have been widely investigated. These experiments have been performed within the framework of a search for the molecular basis of learning and memory; specifically to test the theory that protein synthesis is necessary for the normal operation of memory processes. Protein synthesis inhibiting drugs, including puromycin, CXM, AXM, and Ani have been shown to produce retention deficits in mice and goldfish when administered before training. The impaired retention is usually interpreted as support for the hypothesis that brain protein synthesis is required for some aspect of long term memory consolidation. The same drugs had no effect on acquisition in vertebrates. In contrast, experiments on headless cockroaches indicated that CXM impaired acquisition of the leg lift paradigm, but had no observable effect on retention. The research reported in this dissertation was undertaken to further characterize the effects of CXM on learning and memory in cockroaches. It is important to investigate more fully, especially in insects, these apparently contradictory behavioral effects of a protein synthesis inhibitor to see if this difference is fundamental to the evolution of mechanisms of learning and memory consolidation.

The basic question investigated in this dissertation concerns the specificity of the CXM acquisition impairment effect seen in headless

cockroaches trained in leg lift learning. This question consists of two aspects: (1) is the impairment specific to acquisition in ventral nerve cord preparations or is it also seen in intact animals trained in the leg lift paradigm? (2) is the impairment specific to leg lift training, where peripheral effects of a drug, for example, effects on leg activity, may be very important in performance of the task, or is the impairment also seen in other training situations? The question of specificity of the impairment effect was investigated by 1) replicating the CXM-induced acquisition impairment in headless animals trained to lift a leg,which was previously reported by Kerkut et al. (1970) and Brown and Noble (1967); 2) looking at the effects of CXM on acquisition in intact cockroaches trained in the leg lift paradigm; and 3) investigating the effects of CXM on learning and retention in cockroaches trained in a T-maze. Since the T-maze paradigm is similar to training situations which have been utilized for mice and rats, CXM-produced changes in retention or activity can be compared between an invertebrate and a vertebrate species trained in similar tasks. Protein synthesis inhibition produced in all of these experiments was measured so that a comparison could be made between behavioral effects of CXM and the degree of protein synthesis inhibition.

Another aspect of the investigation of the CXM-induced impairment seen during leg lift training concerns the nature of the impairment effect, i.e. is it a learning or activity effect? Eisenstein (1968) suggested, and Glassman et al. (1970) provided some experimental evidence, that CXM affects performance of the leg lift task. Its effects on actual learning, as proposed by Kerkut et al. (1970) remain to be shown. For this dissertation, several types of experiments

were done to explore this question. (a) In the leg lift experiments, appropriate controls were utilized to try to identify possible CXM effects on leg activity or on the leg's sensitivity to shock. (b) Behavioral experiments were conducted to determine if CXM altered the sensitivity of the leg to shock as reflected in the threshold for initiating twitching in the leg. (c) Electrophysiological experiments were performed in an attempt to assess the effects of CXM on electrical activity of peripheral nerves. While these experiments do not determine the mechanism or site of action of CXM in producing its impairment of acquisition, they do eliminate some possibilities and suggest further experiments.

EXPERIMENTAL METHODS AND RESULTS

I. MEASUREMENT OF PROTEIN SYNTHESIS INHIBITION IN THE COCKROACH CENTRAL NERVOUS SYSTEM

Inhibition by Cycloheximide

The degree of inhibition of protein synthesis produced by the doses of CXM used in the behavioral experiments described below was determined by measuring the incorporation of ^{14}C-leucine into protein of the nervous system. Since two doses and injection techniques were used in behavioral experiments, the inhibition of protein synthesis in both prothoracic ganglion and brain were determined for each dose and technique.

Methods

Adult male cockroaches were obtained from the U. S. Department of Agriculture. CXM was obtained from Sigma Co. and uniformly labelled ^{14}C-leucine (270 mC/mM) from New England Nuclear Corp. CXM was dissolved in insect Ringer solution (0.154 M NaCl, 0.0027 M KCl, 0.0018 M $CaCl_2$, 0.00020 M KH_2PO_4, 0.00047 M Na_2HPO_4) and control animals were injected with the vehicle. Two types of injections were given to different groups after animals were anesthesized with pure carbon dioxide from a tank. (1) Five µl of solution containing 37.5 µg of CXM was injected ventrally into the right side of the prothoracic segment above the prothoracic ganglion, as for training of headless cockroaches. (2) Twenty µl of solution containing 250 µg of

CXM was injected abdominally into the haemocoele near the metathoracic segment, as in training of intact cockroaches. In all experiments, 10 µl of ^{14}C-leucine in 0.01 N HCl (containing 1 µC) was injected abdominally thirty minutes after the injection of CXM or saline. One hour after the leucine injection, the prothoracic ganglion and the brain were dissected out and immediately placed in ice cold insect Ringer solution. The entire dissection procedure took approximately 5 minutes.

Incorporation of labelled leucine into ganglionic and brain protein was determined by the method of Kerkut et al. (1970). The tissue of 10 animals was used in each group. After dissection, the tissue was homogenized in 1.0 ml cold Ringer solution. The homogenate was transferred to a centrifuge tube and 3 ml of 10% trichloracetic acid (TCA) solution was added. The mixture was thoroughly agitated for 30 seconds and centrifuged at 3000 g for 10 minutes. One ml of the supernatant was removed, mixed with 15 ml PCS scintillation fluid (obtained from Amersham/Searle Corp.), and counted on a Packard 3320 liquid scintillation spectrometer. Counting was conducted over a 10 minute period. The pellet was resuspended in 2 ml of chloroform/methanol (1:1) and centrifuged at 3000 g for 5 minutes. The supernatant was discarded and the pellet subjected to a second chloroform/methanol extraction. To the resulting pellet, 1.0 ml of 1 N NaOH was added and the mixture was put in a water-bath at 100°C for 10 minutes. The resulting solution was cooled and centrifuged to remove undissolved material. The supernatant was removed and counted.

Inhibition of protein synthesis was calculated according to the method of Barondes and Cohen (1967). The percentage inhibition was calculated as $(1 - (R_{CXM}/R_{Sal})) \times 100$, where R is the ratio of counts per minute (cpm) of the TCA precipitable fraction (protein) to cpm of the TCA soluble fraction. Since the TCA precipitable fraction contains the labelled leucine which has been incorporated into protein, a reduction in cpm of the TCA precipitable fraction in CXM treated tissue as compared to control tissue indicates a reduction in the amount of protein synthesis occurring between ^{14}C-leucine injection and removal of the tissue.

Results

Table 1 shows the effects of different doses of CXM on ^{14}C-leucine incorporation into protein of the prothoracic ganglion and brain. All doses and injection procedures used in the behavioral experiments produced greater than 90% inhibition of protein synthesis in both the prothoracic ganglion and brain.

These doses did not cause an increase in mortality rate. All headless cockroaches, given either saline or 37.5 µg CXM, lived at least two days following a leg lift training experiment. The animals in both groups gradually died between 2 and 9 days after the experiment. There was also no significant difference in the mortality rate between intact animals injected with saline or 250 µg CXM. Casual observation of animals in both groups revealed no obvious postural or locomotor effects of CXM. Thus doses of CXM which substantially inhibit protein synthesis have no obvious toxic effects in cockroaches.

Table 1. Inhibition of protein synthesis in the cockroach nervous system by different doses of cycloheximide.

Dose	Number of Animals	Substance Injected	Tissue Analyzed	Counts/minute/mg Protein TCA Precipitable	TCA Soluble	Percent Inhibition of Protein Synthesis
-	30	Saline	Prothoracic Ganglion	832.2	18,298	-
-	30	Saline	Brain	1336.6	15,458	-
250 µg	10	CXM	Prothoracic Ganglion	64.1	23,052	94%
250 µg	10	CXM	Brain	67.1	18,000	96%
37.5 µg	10	CXM	Prothoracic Ganglion	55.2	19,344	94%
37.5 µg	10	CXM	Brain	158.5	19,737	91%

Inhibition by Anisomycin

Since anisomycin (Ani) has been used recently as an effective non-toxic protein synthesis inhibitor in mice, the following experiments were done to explore the feasibility of using Ani to investigate effects of protein synthesis inhibition on learning and memory in cockroaches.

Methods

Ani was provided by Nathan Belcher of Pfizer Pharmaceuticals. Solutions were prepared at appropriate concentrations in insect Ringer solution. In order to dissolve Ani, an approximately equal molar amount of HCl was added. In the last experiment, the pH of the solution was adjusted to approximately 7. The dose of Ani injected abdominally into each animal varied from 150 µg to 800 µg. Two schedules of injection were used. The first schedule was the same as used for the CXM experiments, with 90 minutes between the injection of Ani and dissection. In the second schedule, there was an interval of 45 minutes between the Ani injection and dissection, with labelled leucine injected 15 minutes after Ani injection. The methods used to determine the percentage of protein synthesis inhibition produced by Ani was the same as for experiments with CXM.

Results

Table 2 shows the effects of different doses of Ani on ^{14}C-leucine incorporation into protein of the nervous system. The 150 µg dose produced no indication of protein synthesis inhibition. The much larger doses of 600 and 800 µg produced inhibition of about 33%. This is much below the 90-95% inhibition produced by CXM.

Table 2. Inhibition of protein synthesis in the cockroach nervous system by different doses of anisomycin.

Number Of Animals	Dose	Substance Injected	Time Between Injection and Dissection	Tissue Analyzed	Counts/minute/mg Protein TCA-Precipitable	TCA-Soluble	Percent Inhibition of Protein Synthesis
30	-	Saline	90 min	Ganglion	832.2	18,298	-
30	-	Saline	90 min	Brain	1336.6	15,458	-
20	-	Saline	45 min	Brain	740.1	17,838	-
10	150 µg	Ani	90 min	Ganglion	901.0	17,548	none
10	150 µg	Ani	90 min	Brain	1520.8	16,968	none
10	600 µg	Ani	90 min	Brain	958.4	16,502	33%
10	600 µg	Ani	45 min	Brain	495.4	17,748	33%
10	800 µg	Ani	45 min	Brain	481.5	17,414	33%
10	-	Saline*	45 min	Brain	2100.3	26,928	-
10	800 µg	Ani*	45 min	Brain	1675.6	30,349	29%

*pH adjusted to 7

In the last experiment, the pH of the solution was adjusted to about 7.0. This did not appear to influence the degree of protein synthesis inhibition produced by the drug. The procedure for these groups (marked with *) was slightly different than for the other experiments, resulting in somewhat higher cpm of the samples.

Discussion

The inhibition of protein synthesis by over 90% produced by the two doses of CXM was as high as the inhibition of protein synthesis observed in most experiments on vertebrates. CXM-induced protein synthesis inhibition of over 90% was also obtained in the experiments on cockroaches in Kerkut's laboratory (G. W. O. Oliver, personal communication). Thus, experiments in which CXM is administered to cockroaches can be performed to investigate whether synthesis of new protein is necessary for normal operation of learning or memory processes in cockroaches. Of course, it must be remembered that CXM has other physiological effects besides inhibition of protein synthesis.

Ani does not seem to be as effective as CXM in inhibiting protein synthesis in cockroaches. The injections of Ani into cockroaches consisted of much larger doses based on amount of drug per body weight than injections of Ani which produced 90% inhibition of protein synthesis in mice (cockroach doses: 150-800 mg/kg; mouse doses: 25-150 mg/kg). However the experiments on cockroaches indicate that Ani, even in very high doses, does not inhibit protein synthesis to a very large extent, while CXM is an effective inhibitor. Since there is a sheath surrounding the thoracic ganglia and the ganglia in the brain, there may be a difference in the penetrability of the sheath to the two drugs, so that CXM readily gets into the ganglia

while Ani does not. This would be the most likely explanation for the difference in action of the two drugs, especially since Ani has been shown to effectively inhibit protein synthesis in the abdominal ganglion of another invertebrate, Aplysia californica (Castellucci et al., 1972). Further experiments should be done to investigate inhibition of protein synthesis by Ani in cockroaches by injecting Ani directly into the brain to determine if the sheath prevents penetration of Ani into the ganglia.

II. LEG LIFT LEARNING

Cycloheximide Treatment in Headless Cockroaches

Methods

Preparation of Animals. Adult male cockroaches, Periplaneta americana, obtained from the U. S. Department of Agriculture, were kept together in a large bin until the day of the experiment. The cockroaches to be used were removed from the bin and anesthesized with pure carbon dioxide from a tank. Five µl of solution was injected ventrally into the right side of the prothoracic segment above the prothoracic ganglion. The injection consisted either of insect Ringer solution or 37.5 µg CXM dissolved in insect Ringer solution. Each animal was attached with wax by its dorsal surface to a glass rod and decapitated. Vaseline was applied to the neck to prevent the loss of hemolymph. The left prothoracic leg was used for training and the other legs were covered by a strip of gauze to prevent their interference with the left prothoracic leg. Pieces of 0.001" silver wire were tied around the femur and tibia to deliver a shock. A piece of 0.002" silver wire was waxed to the tarsus, with the wax insulating the leg

from the wire. When this leg lead on a P animal made contact with saline solution (1 M KCl) a circuit was completed and shock was delivered to the leg.

During the training period, P and R legs (see page 4 for definition of P and R legs) were wired in series and the tarsal lead of P, upon extension into the saline, initiated shock pulses (50 V, 4/sec) to both P and R legs. These pulses were recorded on a polygraph, as an indication of P's leg position and activity. When the tarsal lead of R made contact with the saline, no shock was given to either leg but the pulses initiated by R were also recorded on the polygraph. During testing the legs were wired in parallel and upon extension each leg initiated shock only to itself. The shocks initiated by each leg during testing were recorded. The circuitry used is shown in Figure 1. Further details of materials and methods used can be found in Eisenstein (1972a).

Experimental Procedure. The animals were injected and wired 1 hour before training and then trained for 45 minutes. After training they were allowed to rest for about 10 minutes and then tested for 10 minutes.

Analysis of Data. Three measures based on the recording of pulses initiated are used to analyze the behavior of the animals. (1) The first is a measure of leg position, i.e. how long the leg remained extended into the solution during a period of time. This is measured by the number of shocks initiated (for the P leg) or by the number of pulses recorded (from the R leg recording circuit), and will be referred to as the number of pulses. (2) The second measure gives information about leg activity by indicating the number

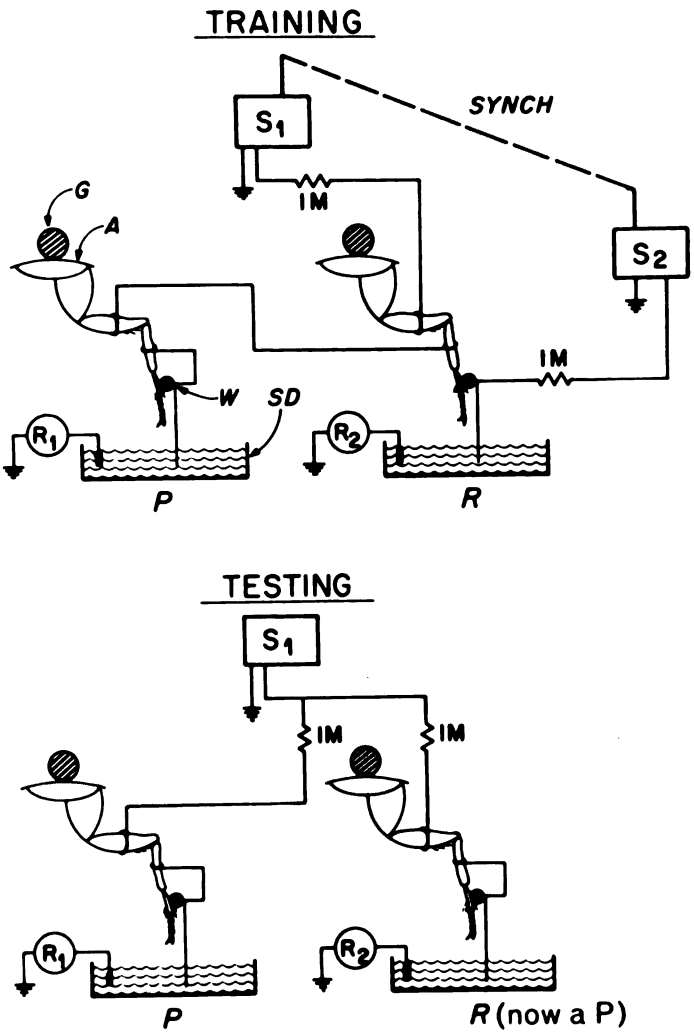


Figure 1. Circuitry and experimental set-up during leg lift training of cockroaches. During training, P and R legs are wired in series and the tarsal lead of P, upon extension into the saline, initiates shock pulses to both legs and these pulses are recorded as an indication of P's leg position and activity. When the tarsal lead of R makes contact with the saline, no shock is given to either leg, but the pulses initiated by R are recorded as for P. During testing the legs are wired in parallel and each leg can, upon extension, initiate shock only to itself. The shocks initiated by each leg are recorded. Abbreviations used: G, glass rod that the cockroach is attached to by its dorsal surface; A, animal; W, wax dab to insulate tarsal lead from the leg; SD, saline dish; S_1, S_2, stimulators; Synch., synchronous frequency output from both P (S_1) and R (S_2) stimulators; R_1, R_2, polygraph recorders for pulses initiated by P and R tarsal leads when they make contact with the saline. Figure from Eisenstein (1970).

of times the leg dips in and out of the solution. The number of times the leg extends into the solution is referred to as the number of dips. (3) The third measure is the dip duration, defined as the number of pulses initiated by an animal for each leg extension. The dip duration indicates how rapidly an animal withdraws its leg from the solution after an extension is made, and for P animals this indicates how rapidly the animal escapes from shock. To calculate this measure for a given time period, the mean of the durations of all dips initiated during that period was determined. A maximum duration was assigned if the duration of the dip was longer than the time period; for example, for a one minute period, in which a maximum of 240 pulses could be initiated, the maximum dip duration was 240 pulses.

An example of the method of counting these three measures is shown in Figure 2.

Results and Discussion

Training. During a 45 minute leg lift training period, P animals rapidly learned to lift their legs while R animals showed great variability in leg position but often kept their legs in an extended position. Figure 3 shows a comparison of learning curves (plotting number of shocks initiated - a measure of leg position) for CXM-injected and saline-injected P animals. The differences between control and CXM-treated groups are significant at the 0.05 level (one-tailed Mann-Whitney U test) for the first 10 minutes; thereafter the differences are not significant. These results indicate that CXM-injected animals take longer to reach an asymptote than control animals, a conclusion also suggested by previous experiments. However, in the experiments reported here, CXM-injected animals begin at a

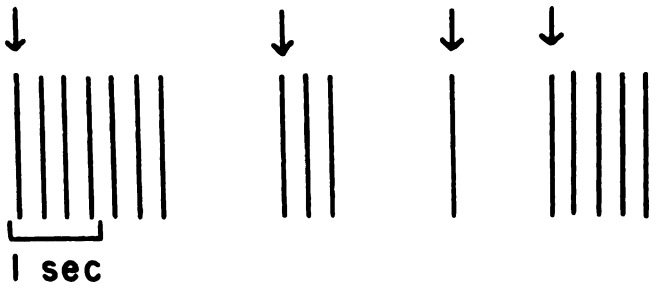


Figure 2. Schematic record of pulses recorded when either P or R tarsal leads make contact with the saline. Pulses are delivered at the rate of 4 Hz. Over a given period of time, the number of pulses initiated is an index of how long the lead is in contact with the saline. How active a leg is, i.e., the number of dips made by the leg in a given length of time, is shown by counting the arrows above the pulses. The mean dip duration for a given period of time is obtained by counting the number of pulses in each dip which begins in that period and calculating the mean. For example, for the period shown in the figure, the mean dip duration would be 4 pulses. The maximum dip duration for any time period is defined as the maximum number of pulses which can be initiated in that period. After the mean dip duration for a given time period was calculated for each animal, the median of the values for all animals in a group was determined for Figures 6 and 10.

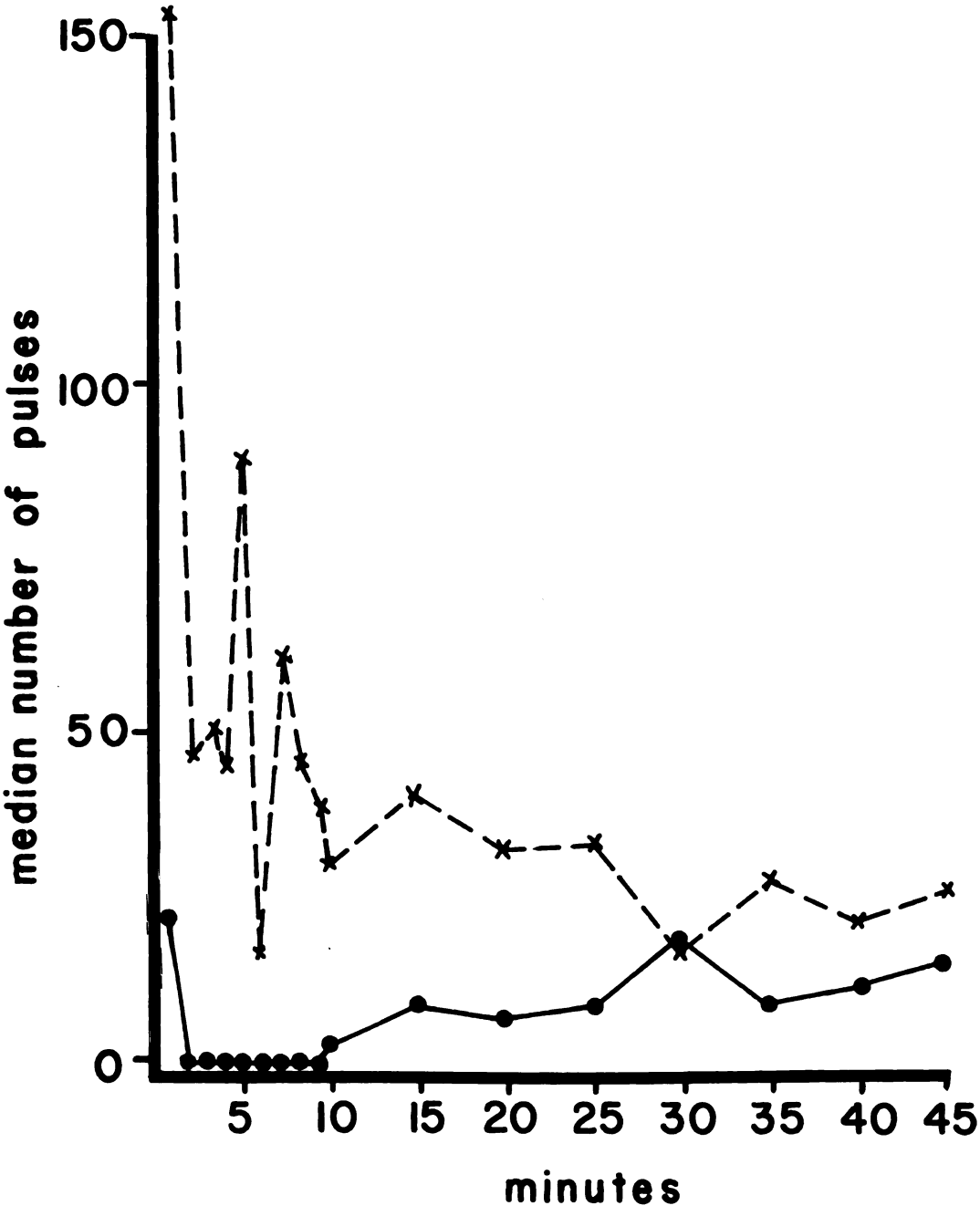


Figure 3. Median number of pulses initiated during a 45 minute training period by headless P animals given saline or CXM before training. This is a measure of leg position. The number of pulses for each minute of the first 10 minutes are shown on the same scale as for the subsequent 5 minute periods. There were 10 cockroaches per group. The differences between the saline and CXM groups are significant at the 0.05 level (one-tailed Mann-Whitney U test) for the first 10 minutes; thereafter the differences are not significant.

much higher level than saline-injected animals, and the difference in the initial level of shocks initiated between CXM and saline groups could account for the CXM-induced impairment of acquisition.

To examine the effects of CXM on the rate of learning, it is necessary to compare the slopes of the learning curves for CXM and saline injected animals. It is difficult to determine an exact slope for the CXM group learning curve (Figure 3) but its initial slope is not obviously different from that of the saline group. These results suggest the possibility that CXM has its effect on acquisition by altering the activity of the leg, making it more difficult for an animal to reach a criterion, instead of directly affecting the learning process. This possibility is supported by the data shown in Figure 5, which depicts the number of dips made by the leg into the solution--an indication of the activity of the leg. The P animals given CXM made significantly more dips in the first 10 minutes of training than the P control animals. Thereafter the differences are not significant. This correlates with the increased number of shocks received by the CXM-injected P animals during the first 10 minutes of training and suggests that the CXM-induced acquisition impairment can be explained by increased leg flexions and extensions of the CXM group. However, this does not rule out other possible effects of CXM which may influence acquisition.

If CXM had a marked effect on leg activity or leg position, the effect should also appear in the R animals, which received the drug and the same amount of shock as P animals, but were not given shock contingent on leg extension. Figure 4 shows the median number of pulses initiated by R animals given CXM or saline. This measure

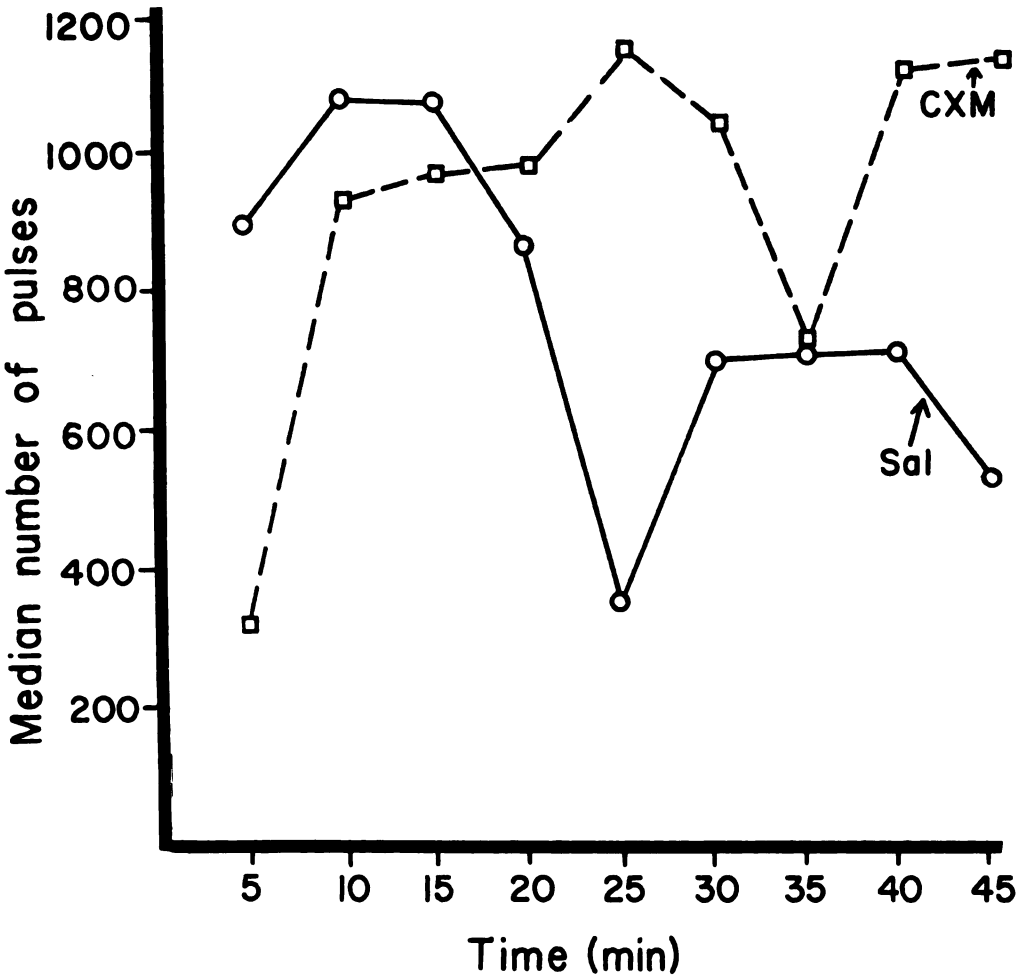


Figure 4. Median number of pulses initiated during a 45 minute training period by headless R animals given CXM or saline. There were 10 cockroaches per group. There were no significant differences between the two groups at any point.

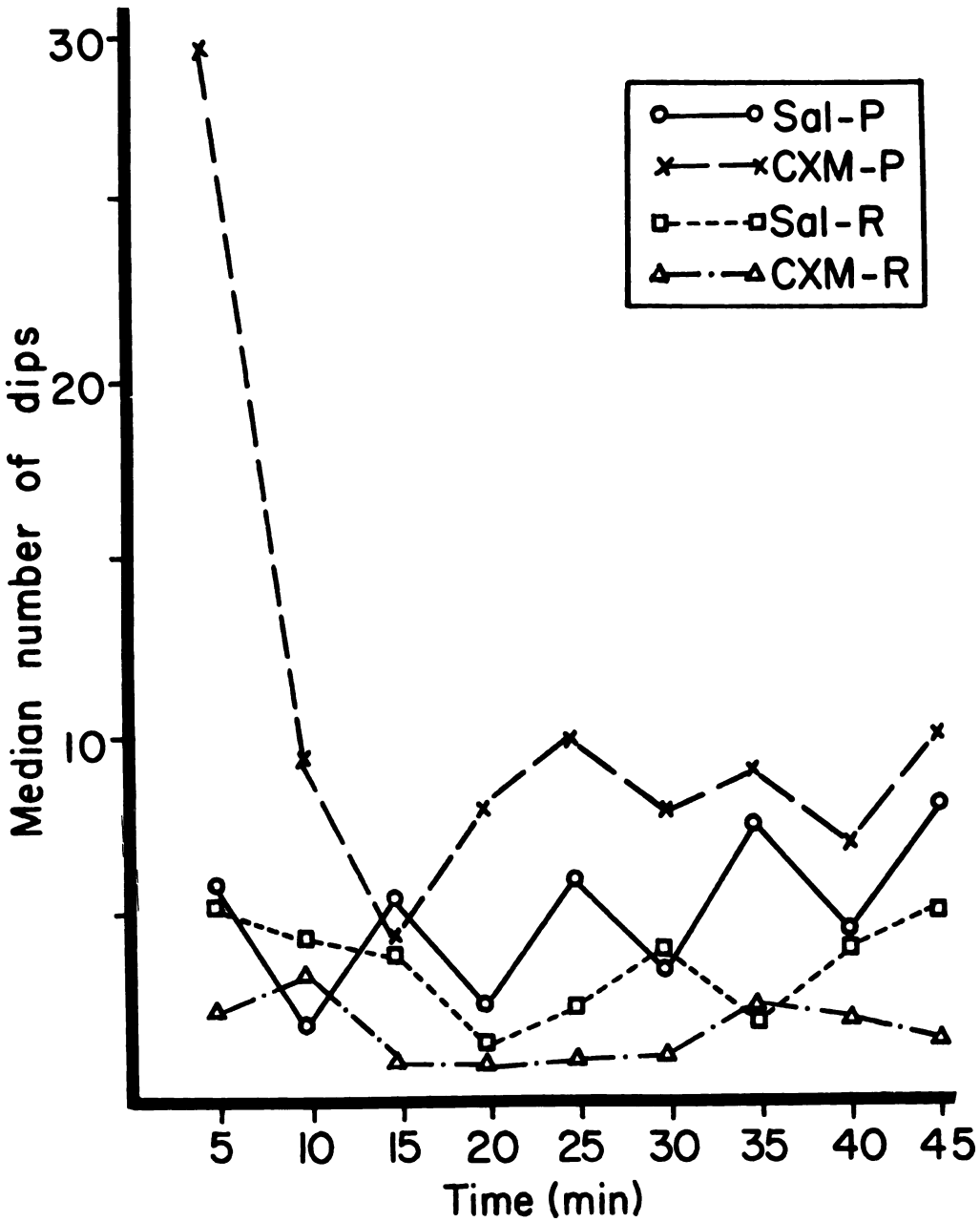


Figure 5. Median number of dips made by headless P and R animals given CXM or saline. The number of dips is an indication of leg activity. There were 10 cockroaches per group. The differences between CXM-injected P animals and saline-injected P animals are significant for the first 10 minutes; thereafter the differences are not significant.

indicates the position (in or out of the solution) of the R leg. Although there is wide variability in the values for the R animals, there is no significant difference between the CXM and saline groups at any point, indicating that CXM does not significantly alter the position of the leg. However, this measure would only reveal a change in position of the leg during training as compared to its position at the beginning of the training period. Although differences between CXM and saline groups in the initial preferred resting position would not be recorded, no marked differences were noted through observation of the animals at the beginning of each experiment.

The leg activity of R animals given CXM or saline can be compared by looking at the measure of number of dips (Figure 5). There is no significant difference in the leg activity between CXM and saline injected R animals, suggesting that CXM alone does not produce a marked increase in spontaneous leg activity. This is somewhat surprising in view of the marked increase in activity seen initially in CXM-injected P animals. The results for R animals would support the hypothesis that CXM affects learning in P animals, rather than leg activity.

In Figure 3, the learning curve for saline-injected P animals shows a sharp drop in the second minute and then a rise during the latter part of training. The rise after 10 minutes is probably due to fatigue of the legs of some animals, which has been seen in other experiments, especially those of Pritchatt (1968). It has not been as prominent in some previous results, possibly because in other experiments the initial activity was higher for the first few minutes

and the learning curves did not reach such a low level in the first few minutes. The amount of fatigue seen probably depends on a number of variables, including duration, magnitude and frequency of shock, and the training demand level (i.e. how far the leg must be raised to escape the shock). Fatigue in just a few animals can affect the average values enough to distort the learning curve, even though learning has definitely occurred in the first 10 minutes.

Examination of changes during training in the dip duration of P animals could indicate if an animal learned to escape shock faster with repeated extensions or if the change in P leg behavior is due entirely to avoidance learning. Figures 6a and 6b show respectively the median dip duration for each minute of the first 10 minutes of training and for each 5 minute period of the 45 minute training period. Due to the method of calculation it is necessary to show separate graphs for 1 minute and 5 minute periods. See page 43 and Figure 2 for methods of calculation. Figures 6a and 6b were obtained by determining the median of the dip duration values for each animal. The curves obtained by calculating the means of the dip durations for each animal in a group show the same trends as the median values, but show more variation.

There is no significant decrease in dip duration over the 45 minute training period for P animals given either saline or CXM (Figure 6b). In the first 10 minute period (Figure 6a), saline-injected P animals show some decrease in dip duration (the decrease between the first 5 minute period and the second 5 minute period is significant at the 0.10 level, one-tailed Mann-Whitney U test). However, this trend does not correspond to the rapid learning seen by the second minute

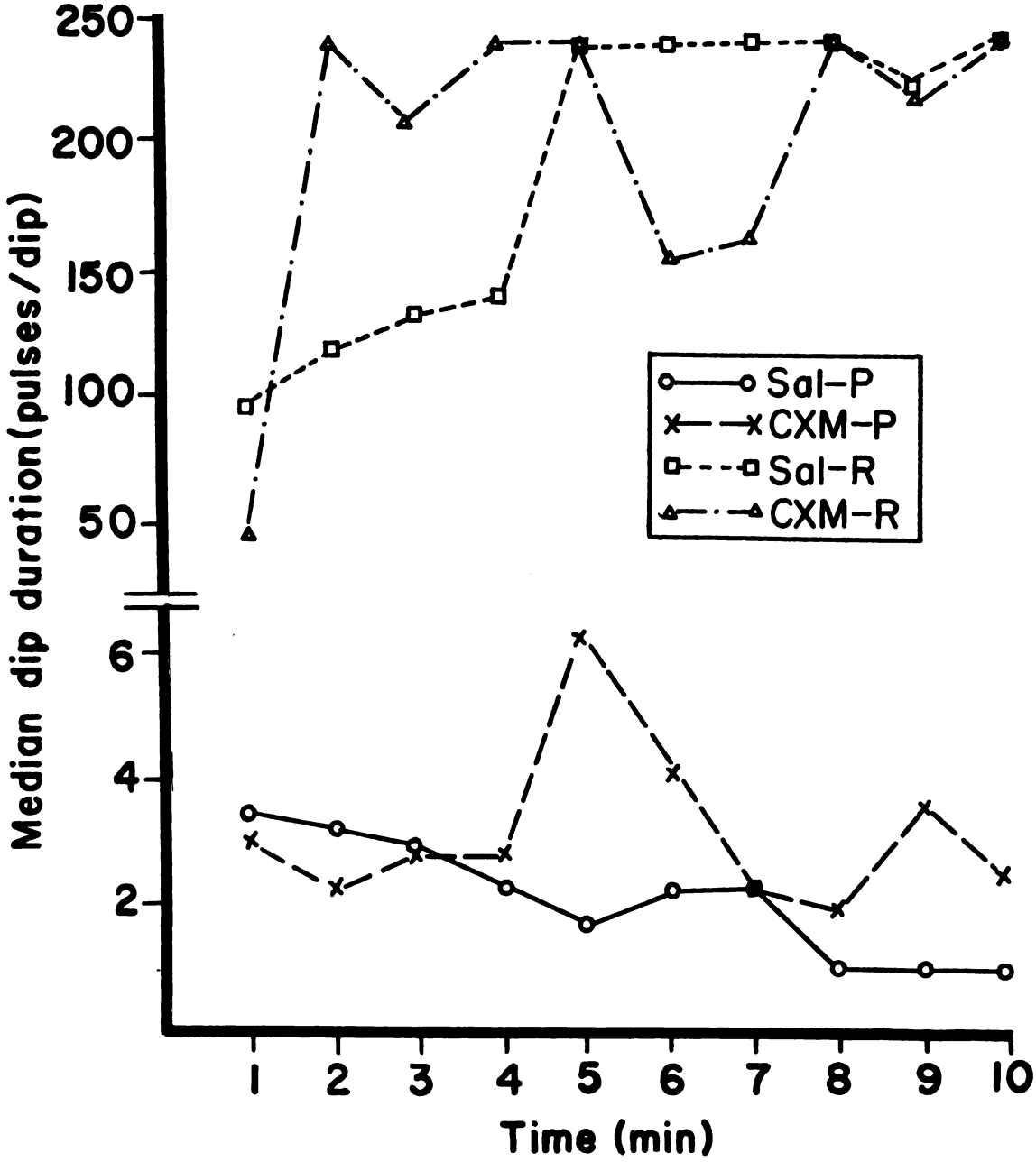


Figure 6a. Median of the average dip duration per animal calculated for each minute of the first 10 minutes of training. The dip duration is an indication of how quickly animals withdraw their legs from the saline solution after extending into the solution. Values are shown on separate scales for headless P and R animals given CXM or saline before training.

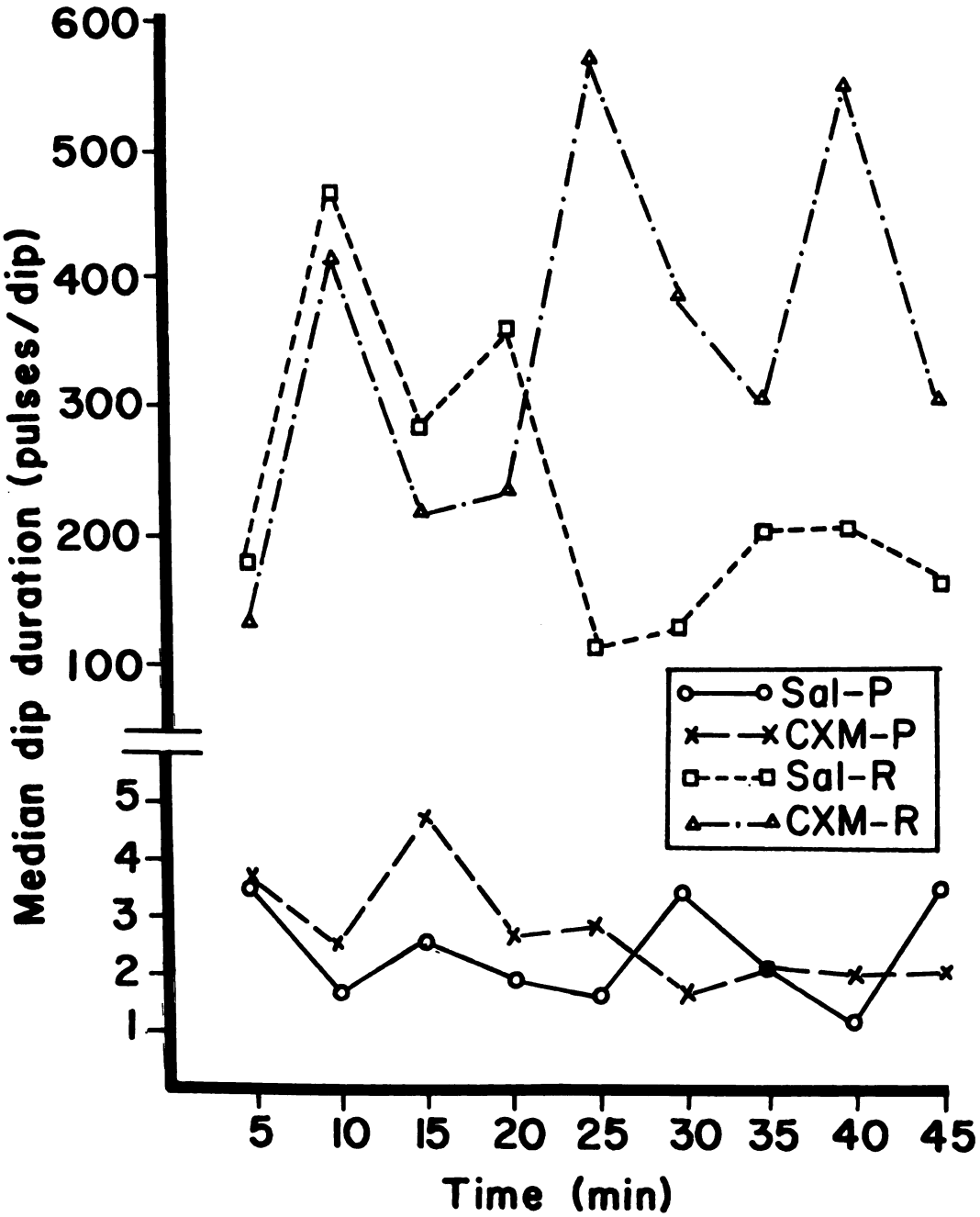


Figure 6b. Median of the average dip duration per animal calculated for each 5 minute period during the 45 minute training period. Values are shown on separate scales for headless P and R animals given CXM or saline before training.

in Figure 3, and thus escape learning apparently does not contribute significantly to the behavior changes represented in Figure 3. Since the saline-P group values for the last several minutes of the 10 minute period (Figure 6a) are determined by a very few animals (as few as two animals made dips in each minute), the reliability of this trend remains to be determined.

There appears to be an increase in dip duration for R animals during the first 10 minutes of training (Figure 6a). However, the increase is not significant and this trend is not maintained during the rest of the training period (Figure 6b).

There is a large difference between the ranges of dip duration values for P and R animals, which would be expected in view of the fact that P animals escape shock by withdrawal from the solution while R animals have no consistent incentive to withdraw their legs from the solution.

There is no significant difference in the dip length between saline-P and CXM-P groups or between saline-R and CXM-R groups.

In summary, several conclusions can be drawn from the above experiments.

1) CXM impairs the rate of acquisition of leg lift training in headless cockroaches (Figure 3). It is possible that the impairment can be attributed, at least in part, to an increase in leg activity of P animals (Figure 5).

2) There is no significant difference between the number of dips (leg activity measure) made by R animals given CXM or saline (Figure 5), indicating that CXM does not produce a marked increase in leg activity of animals given non-contingent shock stimulation.

3) There is no significant difference in the dip duration between saline and CXM groups within a P or R category (Figures 6a,b), suggesting that CXM does not alter the sensitivity of the leg to shock, as reflected in the rapidity of leg withdrawal after shock initiation.

4) There is no reliable evidence that escape learning occurs in this preparation (Figures 6a,b).

Testing. Two indices can be used to determine that learning occurs during leg lift training. The first is a comparison of the P and R learning curves during training, which has been discussed. The second is the difference in behavior between P and former R animals during a testing period in which both animals are retrained as P animals. Early experiments (e.g. Eisenstein and Cohen, 1965) examined both of these indices carefully, confirming that the P animal does learn during the training period. Disterhoft (1972) and Eisenstein (1968) concluded that unless retention is the area of interest, there is no reason to give a test period, as long as simultaneous recording of yoked control R animals is included during training as a check for sensitization. In the experiments reported here, a 10 minute test period was performed soon after training in order to examine the effects of CXM during this procedure.

The mean number of shocks taken by each group during the 10 minute test period is shown in Figure 7. The number of shocks initiated by P animals during the first 10 minutes of training are shown for comparison. First, consider a comparison between P and R animals within each drug group. Although neither the difference between the

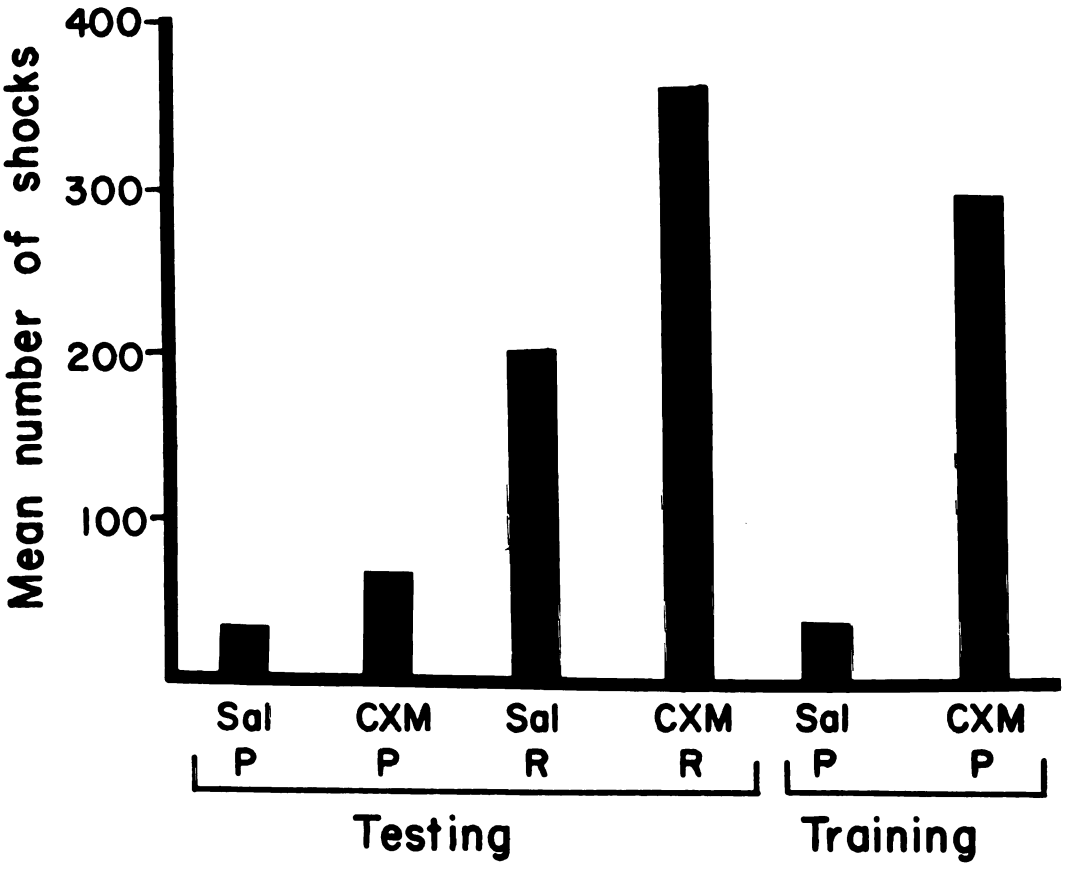


Figure 7. Mean number of shocks taken during a 10 minute testing period by headless P and former R animals given CXM or saline before training. The mean number of shocks taken by headless saline-injected and CXM-injected P animals during the first 10 minutes of training are shown for comparison. There were 10 animals per group. Testing was conducted 10 minutes after the end of training.

saline-injected P and R groups nor the difference between the CXM-injected P and R groups is significant at the 0.05 level, the trends for these groups are in the expected direction, with the R animals taking more shocks during the test period than the corresponding P animals received either during training or during testing. The observation that the former R animal initiates more shocks during testing, when it is being trained in leg flexion, than the P animal initiated during its initial training has been previously reported as a statistically significant event (Disterhoft et al., 1968; Eisenstein and Cohen, 1965). This phenomenon has been interpreted to suggest that R learns during the initial training period that shock may be associated with any leg position and R may have to extinguish that learning before it can learn during testing that leg extension initiates, and flexion avoids, shock.

The amount of P-R difference observed during testing depends on several factors, including (1) the extent to which the shock received by R during training is correlated with extension of the R leg (Disterhoft (1971) showed that the more often shock is received when the leg is extended, the easier it will be to learn at a later time to lift the leg to avoid shock and therefore the less the P-R difference during testing); and (2) the difficulty of the task, which is determined in part by the starting leg position (flexed or extended). In these experiments the level of solution was adjusted so that the lead was in the solution the same amount before testing as before training. Thus the leg position of P and R animals before testing could affect the difficulty of the task to be learned or relearned. For example, P legs may be in a more flexed position at

the beginning of testing since they had learned to flex during the training period. During testing, then, the P legs would have to be lifted even higher to avoid shock, a more difficult task than required of the R legs, which are likely to be in a relatively extended position at the beginning of testing. In this way, good retention by P animals could make retraining more difficult for the P legs, and might reduce P-R differences during testing.

In addition to showing P-R differences, Figure 7 also presents a comparison of the number of shocks initiated by CXM and saline injected groups during testing. Although the differences between CXM and saline P groups and between CXM and saline R groups are not significant during testing, the number of shocks taken by both CXM-P and CXM-R groups is somewhat higher than for the corresponding saline groups, suggesting that the CXM-injected animals take longer to reach a criterion than saline-injected animals. Brown and Noble (1967), using the isolated ganglion preparation, also found that CXM treated animals required significantly longer to reach a criterion during both training and retraining than control animals.

Cycloheximide Treatment in Intact Cockroaches

Methods

The same methods were used to prepare intact animals for leg lift training as for headless animals with the following exceptions.

1) The head was not removed. Antennae were waxed to the rod and wax was placed over the mouth and mouth parts to prevent chewing of the wires attached to the leg.

2) Injections were made by inserting the syringe dorsally and laterally just under the abdominal cuticle and injecting into the haemolymph near the metathoracic segment. The injection consisted of 20 µl of insect Ringer solution or 250 µg of CXM dissolved in 20 µl of insect Ringer. Ten µl of solution was injected on each side of the animal.

3) The animals were trained in a dark room to minimize visual stimuli.

Results

Training. The number of shocks initiated during training by intact P animals injected with CXM or saline is shown in Figure 8. There is no significant difference between the two groups at any point. Acquisition of leg lift training in intact cockroaches is definitely not impaired by CXM; in fact, the trend is in the opposite direction--for CXM injected animals to learn more quickly. Figure 8 also shows the median number of pulses initiated by R animals. As for the headless animals, there is no significant difference between CXM and saline groups.

The median number of dips (indicating leg activity) for all groups is shown in Figure 9. There appears to be a decrease in the number of dips taken by some groups during the training period. There is a significant difference between the number of dips between the first 5 minute period and last 5 minute period for saline-injected P and R animals ($p < 0.01$ for saline-injected P animals; $p < 0.05$ for saline-injected R animals, Wilcoxson two-tailed test). There is no significant difference between the first and last 5 minute periods for CXM-injected P or R animals. As shown in Figure 9, the range of values throughout

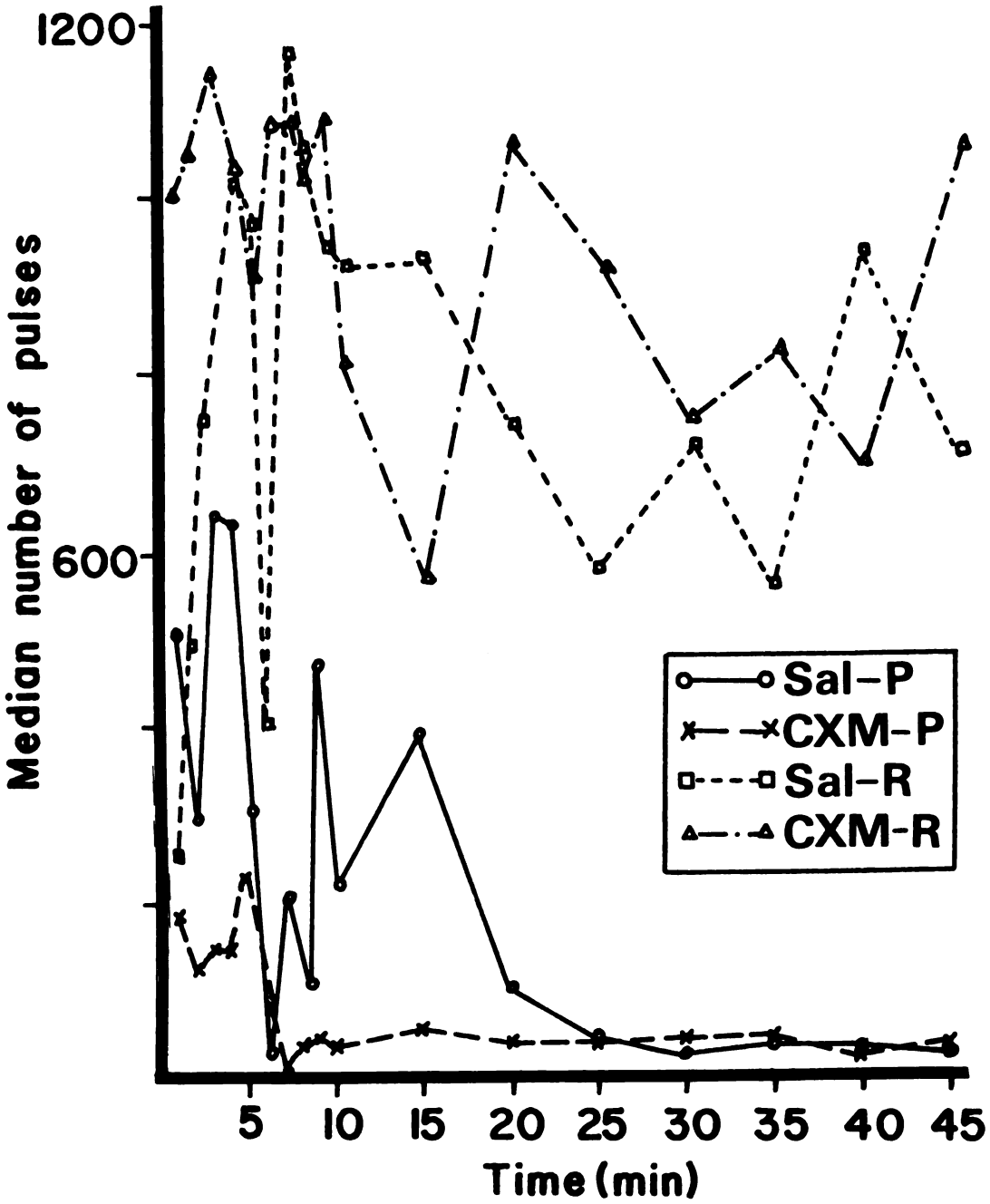


Figure 8. Median number of pulses initiated during a 45 minute training period by intact P and R animals given CXM or saline before training. This measure is an indication of leg position. There were 10 cockroaches per group. The number of pulses for each minute of the first 10 minutes are shown on the same scale as for the subsequent 5 minute periods. The differences between saline-injected and CXM-injected animals within a P or R category are not significant.

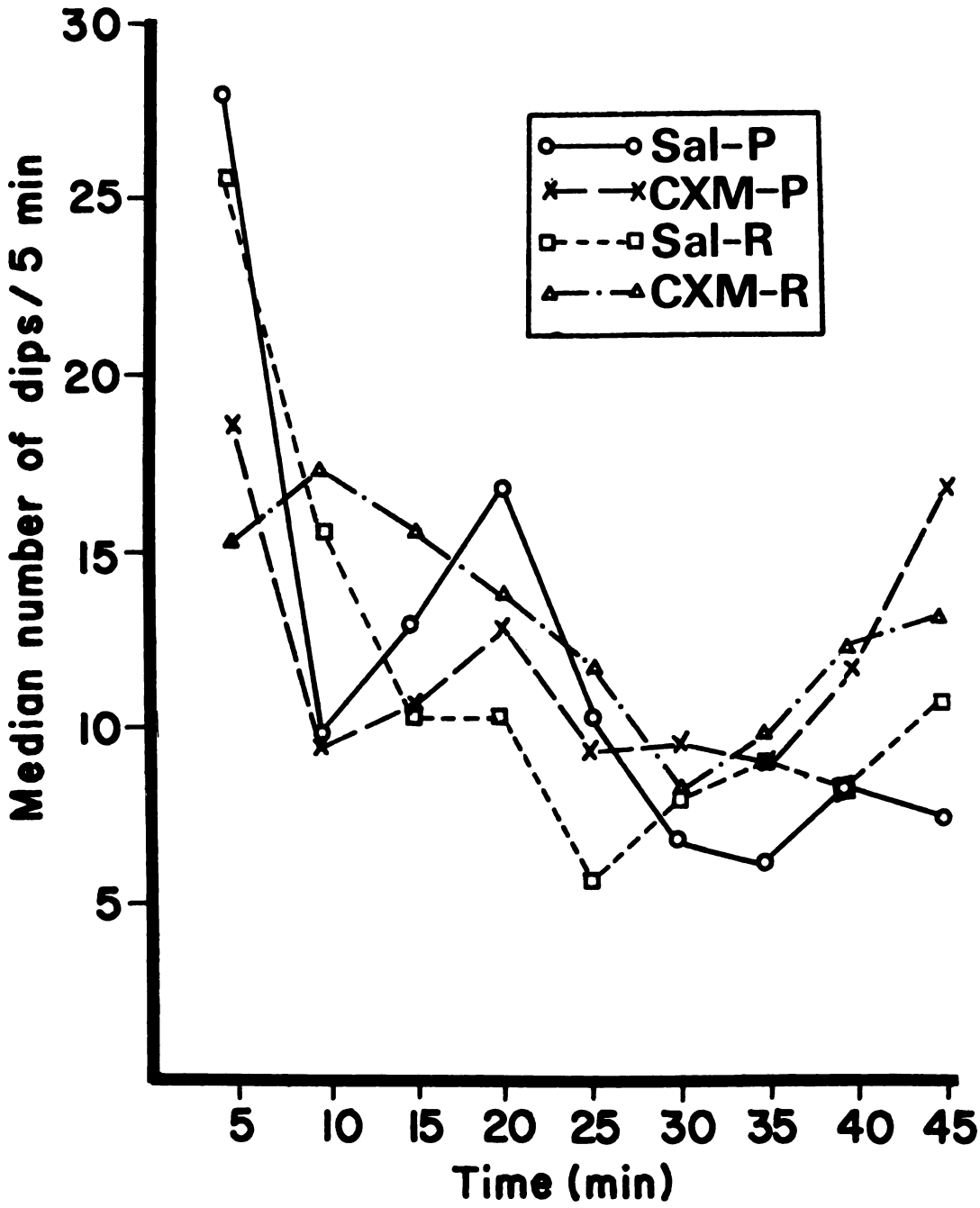


Figure 9. Median number of dips made during a 45 minute training period by intact P and R animals given CXM or saline. The number of dips is an indication of leg activity. There were 10 cockroaches per group. The difference between the first 5 minute period and the last 5 minute period is significant at the 0.01 level for saline-injected P animals and at the 0.05 level for saline-injected R animals (Wilcoxson two-tailed test).

most of the training period is the same for all groups, but saline-injected animals start training at a slightly higher level and end training at a slightly lower level of activity than CXM-injected animals. There is no significant difference between CXM and saline groups at any point, however. The complexity of this phenomenon makes it difficult to draw any meaningful conclusions from the difference in activity trends seen during training between CXM and saline groups. Changes in the number of dips during training are not seen for headless animals, except for the acquisition impairment period seen in the CXM-injected P animals (see Figure 5).

The median dip duration for intact animals is shown in Figure 10. There is no significant difference in dip duration between CXM and saline groups. There appears to be a decrease in the dip duration of P animals during the course of training. If the P animals learned to withdraw their legs from the saline solution more quickly during the training period, then it may be concluded that escape learning has occurred. The change in dip duration between the first 5 minutes and last 5 minutes of training is significant at the 0.05 level for saline-P animals and is significant at the 0.01 level for CXM-P animals (Wilcoxson one-tailed test), suggesting that a component of escape learning is present in learning by intact animals.

Disterhoft (1972) examined the dip duration in experiments training intact cockroaches in the leg lift procedure. He measured the durations of the first 20 dips for each animal and compared the mean of the first 5 dips to the mean of the last 5 dips. He concluded that escape learning did not occur. However, with the recording apparatus used by Disterhoft, very fast dipping actions were not separable, so

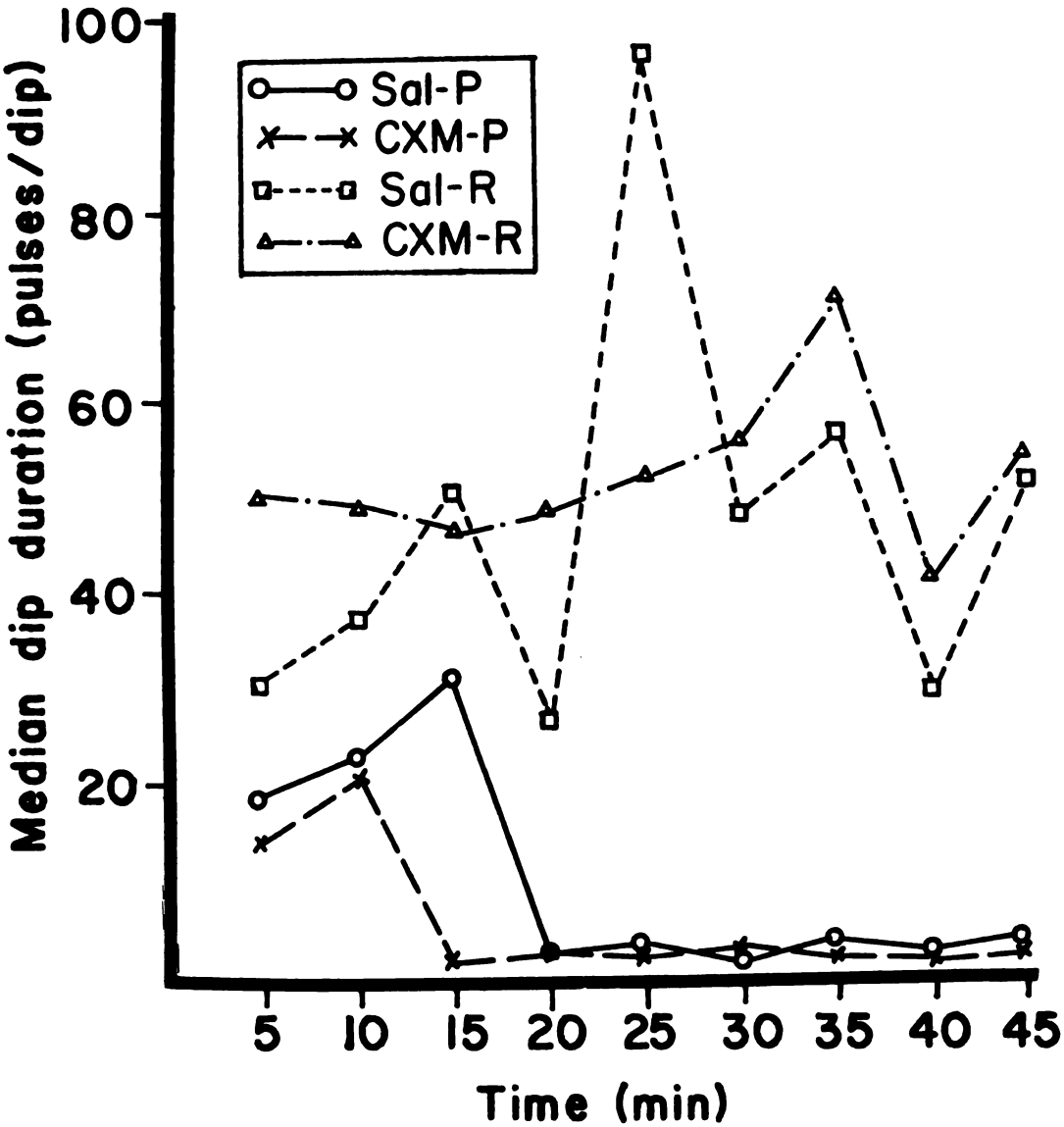


Figure 10. Median dip duration of intact P and R animals given CXM or saline calculated for each 5 minute period of a 45 minute training period. The dip duration is an indication of how quickly animals withdraw their legs from the saline solution after extending into the solution. There were 10 animals per group. The difference between the first 5 minute period and the last 5 minute period is significant at the 0.05 level for saline-injected P animals and is significant at the 0.01 level for CXM-injected P animals (Wilcoxson one-tailed test). This data shows that escape learning occurs in intact P animals trained in the leg lift paradigm.

that more than one dip which could be separated in the present experiments might be recorded as only one dip in Disterhoft's data. In his experiments, the intact cockroaches learned very quickly, reaching an asymptote in about 1.5 minutes, while animals in the present experiment learned much more slowly (see Figure 8). Apparently the learning which occurred in Disterhoft's research is not strictly comparable to the learning which occurred in the present experiments. The reason for this is not clear, since procedures used in the two experiments were not markedly different. In both cases the animals were trained in the dark, although Disterhoft used an enclosed compartment which would have provided more complete darkness and isolation from sensory stimuli than procedures used by the author. Apparently certain variables, as yet unidentified, are of great importance to the animal in determining characteristics of learning and leg behavior.

Comparison of intact and headless groups indicates that there are substantial differences in the learning characteristics of the two preparations. These differences will be discussed in the main discussion section (see page 111).

In summary, the following conclusions emerge from results of studies of intact cockroaches given leg lift training after CXM injection.

1) CXM does not impair acquisition of leg lift training in intact cockroaches.

2) There is no significant difference between saline and CXM groups trained as P animals, or between saline and CXM groups treated as R animals, on any behavioral measure that was analyzed.

3) Intact cockroaches appear to show escape learning in addition to avoidance learning.

Testing. Figure 11 shows the mean number of shocks taken by all groups during the 10 minute test period. Due to technical difficulties, only 6 pairs in the saline group and 8 pairs in the CXM group could be used for testing data. The mean number of shocks initiated by P animals during the first 10 minutes of training is shown for comparison. The difference between saline P and R groups is significant at the 0.02 level (one-tailed Mann-Whitney U test) and the difference between CXM-injected P and R groups is significant at the 0.10 level. Thus both P-R differences in learning curves (see Figure 8) and P-R differences in testing confirm that intact P animals learned during training.

The differences during testing between saline-injected and CXM-injected P animals and between saline and CXM injected R animals are not significant at the 0.05 level. This lack of difference between intact saline and CXM groups during testing is consistent with the lack of difference seen between saline-injected and CXM-injected animals during training. Also, no difference between CXM and saline groups was observed during testing of headless animals (Figure 7).

The testing results reported for intact animals are not strictly comparable to those for headless animals, because the level of the saline solution was not adjusted between training and testing for intact animals (since the experiment was conducted in the dark). Thus the values shown on the ordinates during testing (Figures 7 and 11) should not be compared for intact and headless preparations. Horridge (1962) demonstrated that either testing procedure (i.e. adjusting

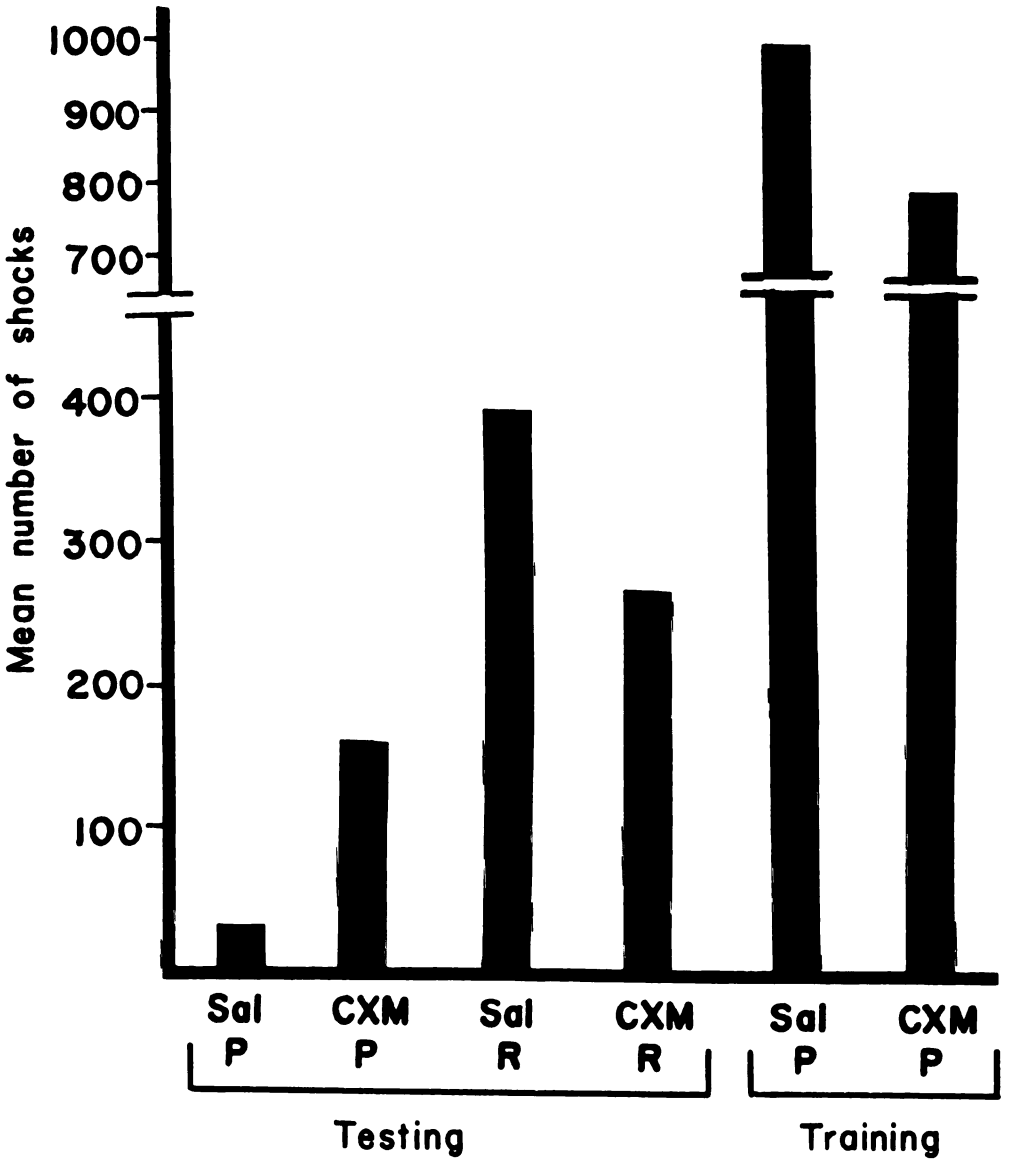


Figure 11. Mean number of shocks taken during a 10 minute testing period by intact P and former R animals given CXM or saline before training. The mean number of shocks taken by intact saline-injected and CXM-injected P animals during the first 10 minutes of training are shown for comparison. There were 8 CXM-injected pairs used for testing and 6 saline-injected pairs used for testing. Testing was conducted 10 minutes after the end of training. The difference between saline-injected P and R groups during testing is significant at the 0.02 level (one-tailed Mann-Whitney U test) and the difference between CXM-injected P and R groups is significant at the 0.10 level. The differences between saline-injected and CXM-injected animals within a P or R category are not significant.

the level of the solution between training and testing or not adjusting the level) could be reliably used to determine P-R differences during testing.

The data shown in Figure 11 indicates that fewer shocks were initiated by former R animals when they are being trained to lift a leg during testing than were initiated by P animals during initial training. This is in contrast to the situation for headless animals (Figure 7), where former R animals initiated more shocks during testing than P animals did during training. This phenomenon for intact animals has not been previously reported. Apparently the random shock experience received by intact R animals during the training period makes it easier for them to learn at a later time that extension initiates shock and flexion avoids shock, while a similar experience received by headless R animals during training makes it more difficult for them to associate shock with leg extension during testing. This could indicate a basic difference in the association processes mediated by the brain and by the ventral nerve cord.

III. T-MAZE TRAINING EXPERIMENTS

Characteristics of T-Maze Learning in Cockroaches

In these experiments cockroaches were trained to turn either right or left in a T-maze using electric shock as punishment for an incorrect response.

Methods

Apparatus. A diagram of the T-maze is shown in Figure 12. The maze was constructed of plexiglas. The runway was completely transparent; the walls of the arms were opaque and the ceiling was transparent.

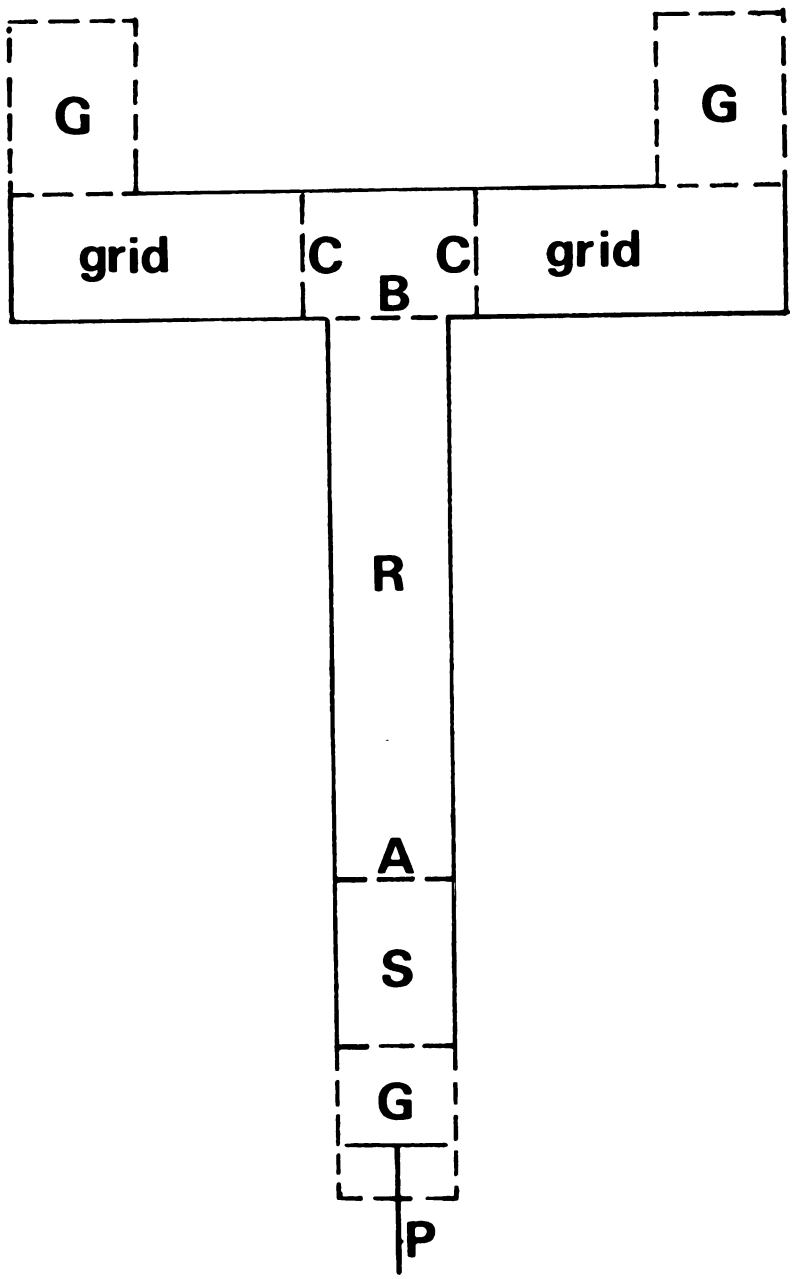


Figure 12. Top view of the T-maze used for training cockroaches. The dotted lines indicate sliding doors which can be raised or lowered. All goal boxes contain a plunger and can be placed either at the start box or at the end of either arm of the maze. The floors of the arms are covered with a shock grid. The maze, constructed of plexiglas, is 22 cm long (excluding the goal box) and 19 cm wide across the arms. The runway is 3.2 cm wide and 3.8 cm high. See text for further details and training procedures. Abbreviations used: G, goal box; P, plunger; S, start box; R, runway; A, B, C, sliding doors.

The goal boxes were totally opaque and provided a small dark enclosure, which is usually attractive to a cockroach. The shock grids on the floor of the arms were made by photoetching copper film on a glass epoxy backing to form 2 mm wide strips separated by 0.8 mm. The grids were gold plated to prevent oxidation. The source of the shock was 60 cycle AC current reduced to approximately 8 V with a variable transformer. The amplitude was set so that the shock caused immediate escape from the incorrect arm, but did not produce convulsions or noticeable erratic behavior.

Training Trials. At the beginning of a training trial the animal was in the goal box which was then placed at the entrance to the start box. The animal was pushed into the start box with a plunger. He remained in the start box for 15 seconds, after which the door to the runway (A in Figure 12) was raised. If the cockroach did not leave the start box within 20 seconds, he was gently prodded. After he proceeded down the runway and into one of the arms, door C on the appropriate side was closed. If the animal entered the wrong arm he turned around when the shock was received and was allowed to enter the correct arm. The animal usually entered the dark goal box of the correct arm immediately and the box was closed. In most cases, animals proceeded spontaneously down the runway and into a goal box, but occasionally they had to be prodded with a brush at some point. After entering the goal box, an animal remained there for 2 minutes, at which time the goal box was placed at the entrance to the start box and another trial began.

Experimental Procedure. Cockroaches obtained from the U. S. Department of Agriculture were housed together with access to dog

food and water until the day of the experiment. A cockroach was removed from the colony, placed in a goal box for 2 minutes and given a brief (usually 1-2 minutes) exposure to the T-maze; i.e. he was allowed to wander into both arms of the maze. He was then removed from the maze and injected. The injection consisted of 20 µl of insect Ringer solution or 20 µl of Ringer solution containing 250 µg of CXM. The solution was injected abdominally using the same method as for intact animals trained in the leg lift. The training procedure began 45 minutes after injection.

The training procedure consisted of a free trial on which no shock was given and 20 training trials in which shock was administered in one arm of the maze. An animal was trained to turn opposite to the direction he chose on the first training trial, so that he always made an incorrect choice and received shock on the first training trial. If an animal had preference for turning one direction, he would be trained against this preference.

Animals were tested for retention at 5 minutes, 1 hour, 5 hours, or 22 hours after the end of the training period. The retention test consisted of 10 training trials--no free trial was given. Cockroaches were kept in individual containers in the dark between training and testing.

Data Analysis. During the course of training, the following three measures were recorded on each trial in addition to the direction turned (these measures give an indication of the activity of the animals in the maze):

1) start box time--time spent in the start box after door A (Figure 12) was raised ;

2) runway time--the time taken by the animal to proceed down the runway to the location of door B ;

3) choice time--time taken by the animal from the end of the runway until he entered the correct goal box

Results and Discussion

Cockroaches learned to turn right or left in a T-maze to a criterion of about 80% in one training session of 20 trials. Figure 13 shows the percentage of correct responses of 32 saline-injected animals for each trial during the 20 trial training session. A control group was given the same treatment except that no shock was given in the maze (the animal could turn either direction and enter a goal box). In this group the direction turned on the equivalent of the first training trial was counted as "incorrect" and the direction turned on the succeeding trials was scored as being "incorrect" (for the same direction) or "correct" (for the opposite direction). The fraction of correct choices was less than 50% on most trials, suggesting that the animals may have a slight preference for the direction turned on the first training trial. The "no shock" control group showed that the tendency of the trained animals to enter the correct arm more often as training progresses is produced entirely by the training shock and not by prolonged exposure to the maze.

The animals showed a significant tendency to proceed down the runway more slowly as training progresses (see Figure 16). This phenomenon was seen in both trained and "no shock" groups and in both CXM-injected and saline-injected groups. A comparison of the mean of

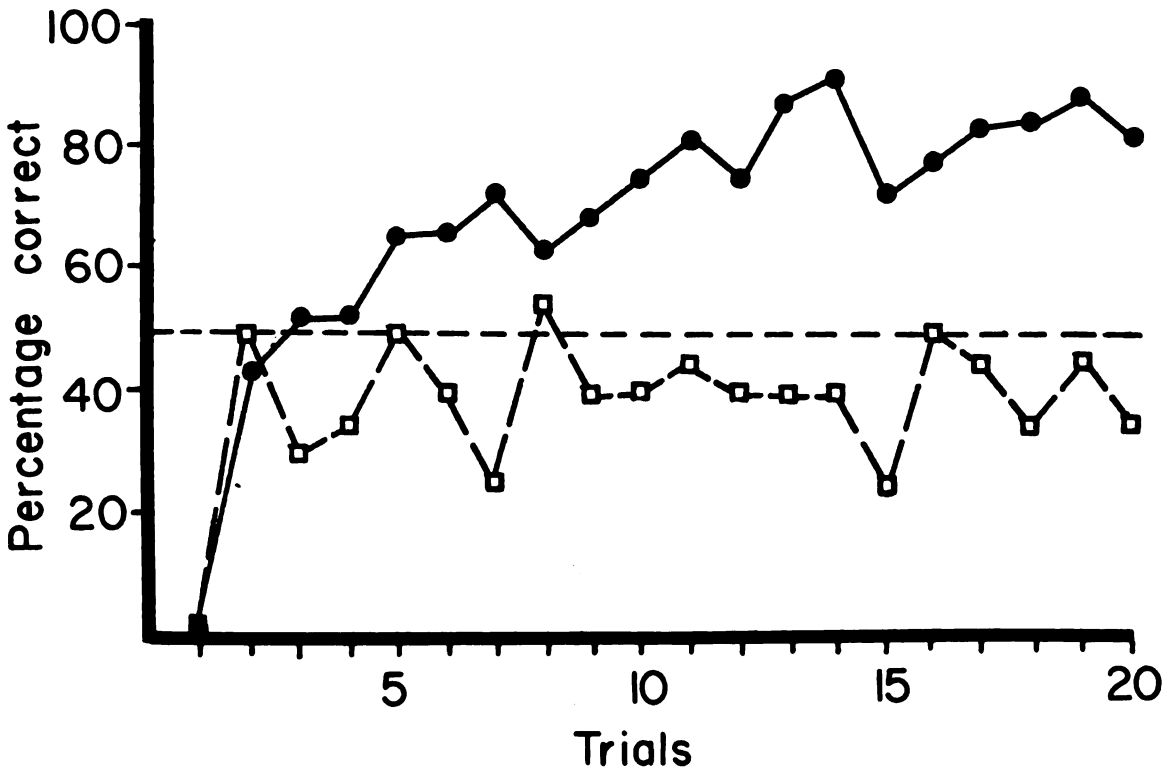


Figure 13. Percentage of correct turns made by cockroaches in a T-maze. The trained saline-injected group (represented by closed circles; 32 animals) was trained to turn left or right to avoid shock. Animals in the "no shock" group (represented by open squares; 20 animals) were given the same experience in the maze as the trained group except that shock was not administered and they could turn either direction to reach a goal box. The dotted line shows the 50% level of correct choices which would be expected by chance. The "no shock" control group confirms that learning actually occurred as a result of shock administration.

trials 1-4 and the mean of trials 17-20 showed a significant difference ($p < 0.002$ for each group, two-tailed Mann-Whitney U test). The difference in runway time between trained and "no shock" groups was not significent. Thus, the increase in runway time during 20 training trials would appear to be the result of exposure to the maze rather than a shock-mediated phenomenon.

Retention of the learned behavior was evident up to 22 hours after training, although there appeared to be a slight decrease at 22 hrs (see Figure 15 and Table 4). There was no correlation between the degree of acquisition as measured by the number of correct trials during training, and the amount of retention, as measured by the number of correct trials during testing. These two indices were plotted against each other for each individual animal in Figure 14 and no correspondence could be seen. This suggests that the length and amount of retention are independent of the characteristics of acquisition of a task, a result which has been observed in mice (Squire and Barondes, 1972).

The use of a "free trial" preceding training permitted the observation of spontaneous alternation. Spontaneous alternation, which is commonly seen in mice and rats, has been described as the non-appetitive, sequential alternation of right and left turning responses on repeated trials at a level of significance greater than chance. In 44 cockroaches injected with saline solution (including trained and "no shock" groups), 22 chose the same arm on both the free trial and the succeeding trial. Out of 40 animals injected with CXM, 20 chose the same arm on the first two trials. Thus the choice made on the second trial was the same as for the first trial 50%

Table 3. Percentage of cockroaches reaching a criterion of 5/6 correct turns in a T-maze within 20 training trials during training or within 10 training trials during testing.

Substance Injected	Training		Testing	
	Percent Achieving 5/6 Correct	Total Number Trained	Percent Achieving 5/6 Correct	Total Number Tested
Saline	90%	32	82%	28
CXM	93%	32	91%	24

Table 4. Mean number of trials required for cockroaches in each retention group to reach a criterion of 5/6 correct turns in a T-maze during training and during testing.

Substance Injected	Retention test Interval	Number of Animals	Mean Number of Trials to reach 5/6 Correct	
			Training	Testing
Saline	5 min	8	11.9	7.7
Saline	1 hr	8	9.6	6.4
Saline	5 hr	6	10.2	6.5
Saline	22 hr	6	13.7	8.2
Saline	All	28	11.3	7.2
CXM	5 min	6	12.5	6.2
CXM	1 hr	6	12.6	6.7
CXM	5 hr	6	12.0	6.3
CXM	22 hr	6	11.3	7.7
CXM	All	24	12.1	6.7

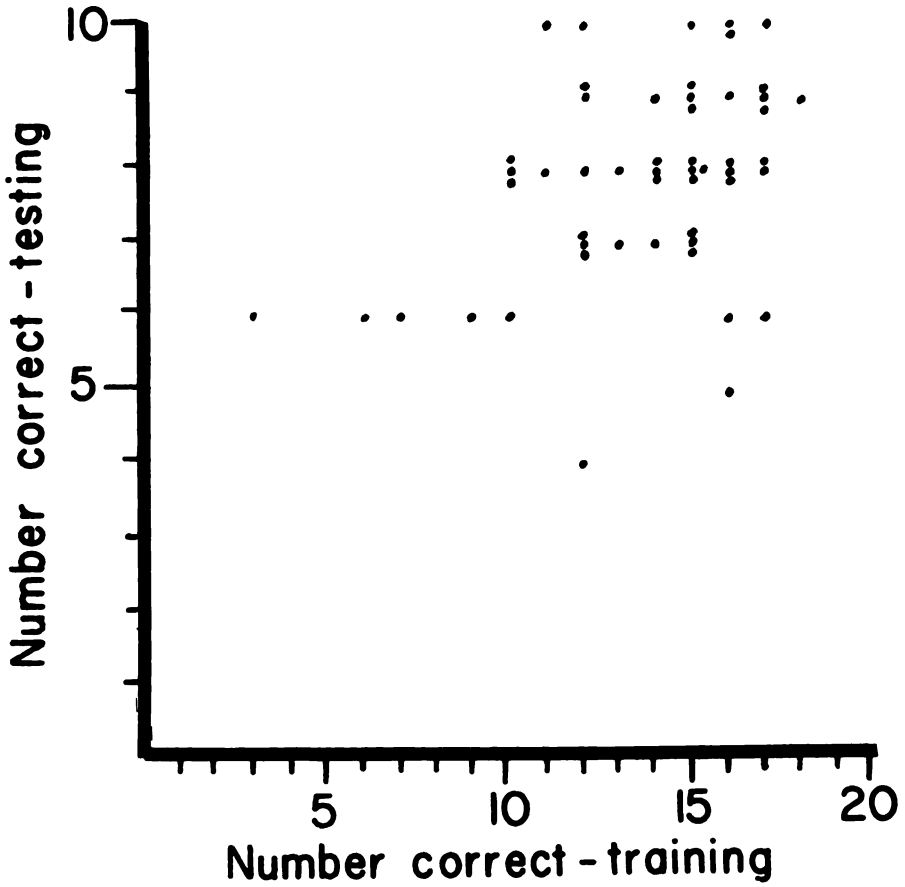


Figure 14. The number of correct trials during training is plotted against the number of correct trials during testing for each individual cockroach -- a total of 52 animals. The random scatter shows that the amount of retention displayed by an animal during testing is independent of the amount of acquisition displayed during the training period.

of the time, the result expected by chance. This suggests that cockroaches in this apparatus do not spontaneously alternate, but instead choose the arm to be entered on each trial at random.

Examination of the number of spontaneous alternations made throughout 21 trials by the "no shock" group supports this conclusion. Each combination of two succeeding trials was scored as a spontaneous alternation if the two choices were different. Each animal provided 20 2-trial combinations and there were 20 animals given "no shock" treatment. Of these, 12 were injected with saline and 8 with CXM, but the two groups showed no difference in alternation behavior, and will be combined for data analysis. Of the 400 2-trial combinations, 54% were classified as spontaneous alternations, a result no different than expected by random choice in the maze.

There are several factors in this situation which could interfere with a tendency to show spontaneous alternation. On the free trial, entrance into the goal box could act as a reward and influence the direction turned on the subsequent trial. Also, the maze was designed so that the animals make two turns on each correct trial--one turn into the arm and a second turn in the opposite direction to enter the goal box. Thus a tendency to alternate based on turning direction (i.e. left or right) would conflict with a tendency to alternate based on alternation of stimuli in the two arms. Data from rat behavioral experiments demonstrates that rats exhibit spontaneous alternation behavior and strongly suggests that rats do not simply alternate turns, but instead alternate stimuli (Dember and Fowler, 1958), but there is no data available for cockroaches which analyzes

spontaneous alternation behavior. The experiment reported here should detect spontaneous alternation behavior in cockroaches based mainly on alternation of stimuli, but no alternation behavior was seen. Thus the existence of spontaneous alternation behavior in cockroaches remains to be demonstrated.

Cycloheximide Treatment in Cockroaches Given 20 Training Trials

Figure 15 shows a comparison of learning curves for saline-injected and CXM-injected animals trained to turn left or right in a T-maze. There were 32 animals in each group. There is no significant difference in the characteristics of learning between the two groups. Thus a dose of CXM which inhibits protein synthesis by over 90% does not cause impairment of T-maze learning.

The amount of retention, as measured by relearning, demonstrated by animals tested at various times is also shown in Figure 15. There were 8 animals in the saline-injected groups tested at 5 minutes and 1 hour, and 6 animals in all other groups. The number of correct trials during testing was as high or higher for CXM groups compared to saline groups at all retention intervals. There was no significant difference among any of the retention groups. Thus CXM does not cause a retention deficit up to 22 hours after training in this situation.

The conclusion that CXM does not impair learning or memory in this paradigm is supported by looking at the number of trials taken by animals to reach a criterion of 5 correct out of 6 consecutive trials (5/6 correct). If an animal did not achieve this criterion

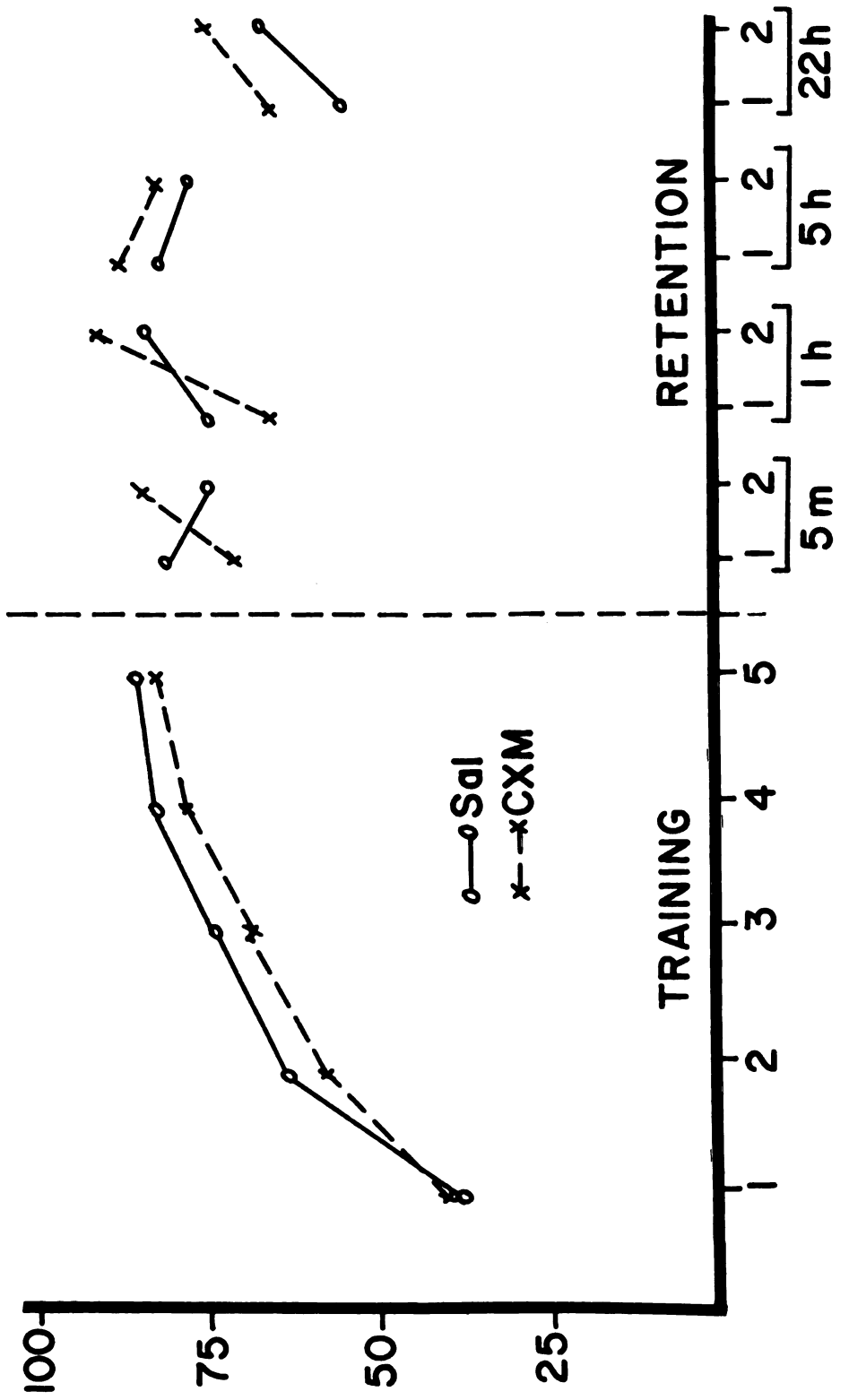


Figure 15. Percentage of correct turns in blocks of 4 trials made by cockroaches trained to turn left or right in a T-maze after injection of CXM or saline. There were 32 animals in each group. The percentage of correct turns made by cockroaches tested at one of four retention intervals is also shown. There were 8 animals in the saline-injected 5 minute and 1 hour groups and 6 animals in all other retention groups. There was no significant difference between CXM and saline groups at any point during training or testing.

in 20 trials, a value of 21 was assigned. Most animals did reach the criterion (Table 3). The mean number of trials required for animals in each retention group to reach a criterion of 5/6 correct is shown in Table 4.

Although CXM did not inhibit learning or retention of cockroaches trained in the T-maze, the drug did increase the activity of the animals in the maze. The mean runway time during training, one indication of the activity level, is shown in Figure 16. Since no significant difference existed between trained and "no shock" animals within each drug group, the animals will be treated in two groups--saline injected and CXM-injected. CXM-injected animals have a significantly lower average runway time than saline-injected animals ($p < 0.0001$, two-tailed Mann-Whitney U test). Both CXM and saline groups demonstrate a marked increase in runway time during the training period. There is a significant difference between the mean runway time of trials 1-4 and the mean runway time of trials 17-20 ($p < 0.001$ for each group). Thus CXM reduced runway time without affecting the basic phenomenon of increase in runway time during the training period.

The time spent in the start box after the door to the runway was raised was not significantly different for the drug and control groups and there was no change during the course of training. After 20 seconds in the start box, the animals were prodded gently with a brush to get them to leave. The majority of animals in both groups did not leave the start box spontaneously within 20 seconds and thus had to be prodded. The CXM-injected animals seemed more sensitive to this prodding than control animals and started much more quickly

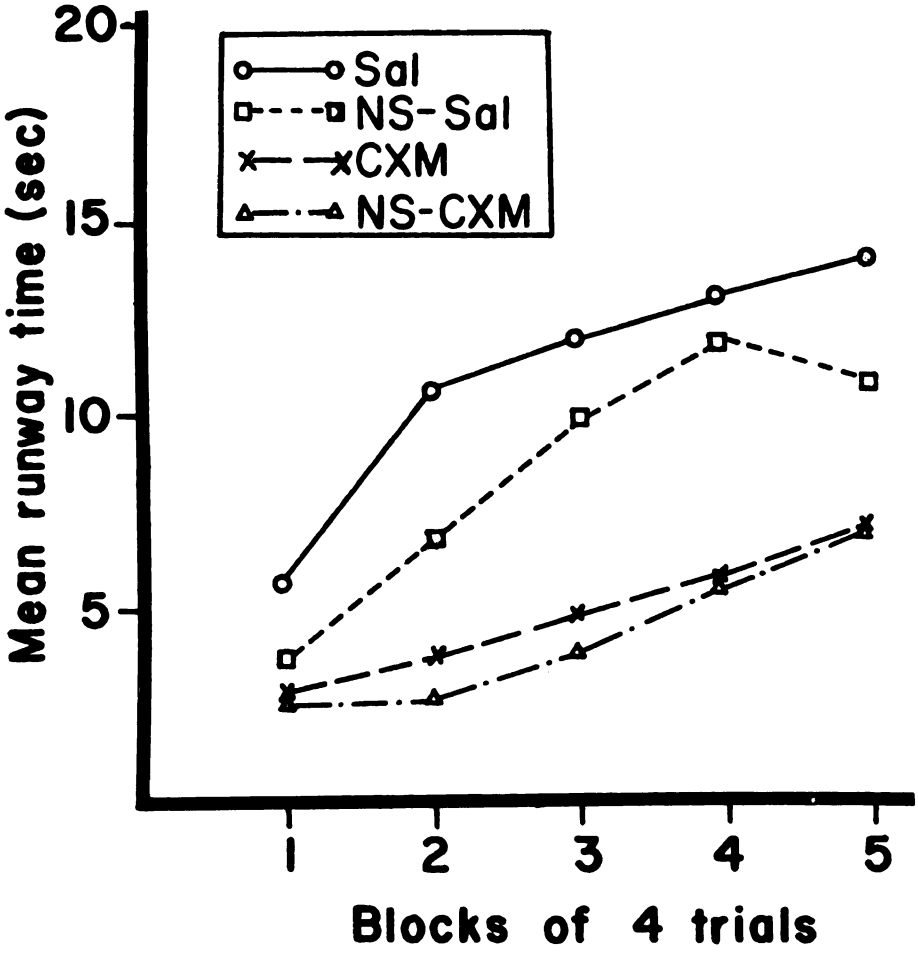


Figure 16. Mean runway time during training in blocks of 4 trials for saline-injected and CXM-injected cockroaches given T-maze training or maze exposure with no shock. The runway time is an indication of the activity of the animals in the maze. There were 32 animals in the saline-injected and CXM-injected trained groups, 12 animals in the saline-injected "no shock" group, and 8 animals in the CXM-injected "no shock" group. There was no significant difference between trained and "no shock" animals within each drug group. CXM-injected animals had a significantly lower average runway time at each point than saline-injected animals ($p < 0.0001$, two-tailed Mann-Whitney U test). There was a significant difference between the mean runway time in trials 1-4 and the mean runway time in trials 17-20 for both CXM and saline groups ($p < 0.001$ for each group). Thus CXM reduced the runway time without affecting significantly the basic phenomenon of increase in runway time during the training period.

after being prodded. The CXM induced decrease in runway time could be due either to a decrease in sensory threshold or to a spontaneous increase in locomotor activity, and the observed response to prodding suggests a major role for the first alternative.

The time taken to enter the goal box after reaching the choice point (choice time) was reduced in CXM-injected animals (Figure 17). This measure is a rather complex one to interpret since it consists of three components: 1) how long the animal took to make a decision about which arm to enter; 2) how long the animal took to leave the shock grid and enter the correct arm if an incorrect choice was made; and 3) how long the animal took to enter the goal box after reaching the correct arm. Unpredictable behavior shown by individuals in one of these three areas often made the choice time a rather imprecise measure. Analysis of the choice time, shown in Figure 17, confirms the observation that CXM increased activity of the animals. Considering only the trained animals, there was no significant difference between the means of trials 1-4 for CXM-injected and saline-injected animals. However there was a highly significant difference ($p < 0.0001$, two-tailed Mann-Whitney U test) between the means of trials 17-20 for CXM and saline groups. This change appears to be mainly due to an increase in the time to enter the goal box shown by saline animals during the course of training. CXM animals do not show the increase during training for this measure. It is not clear why CXM and saline groups do not show the same trends during the training period for the choice time measure while they do show the same trends in the runway time measure.

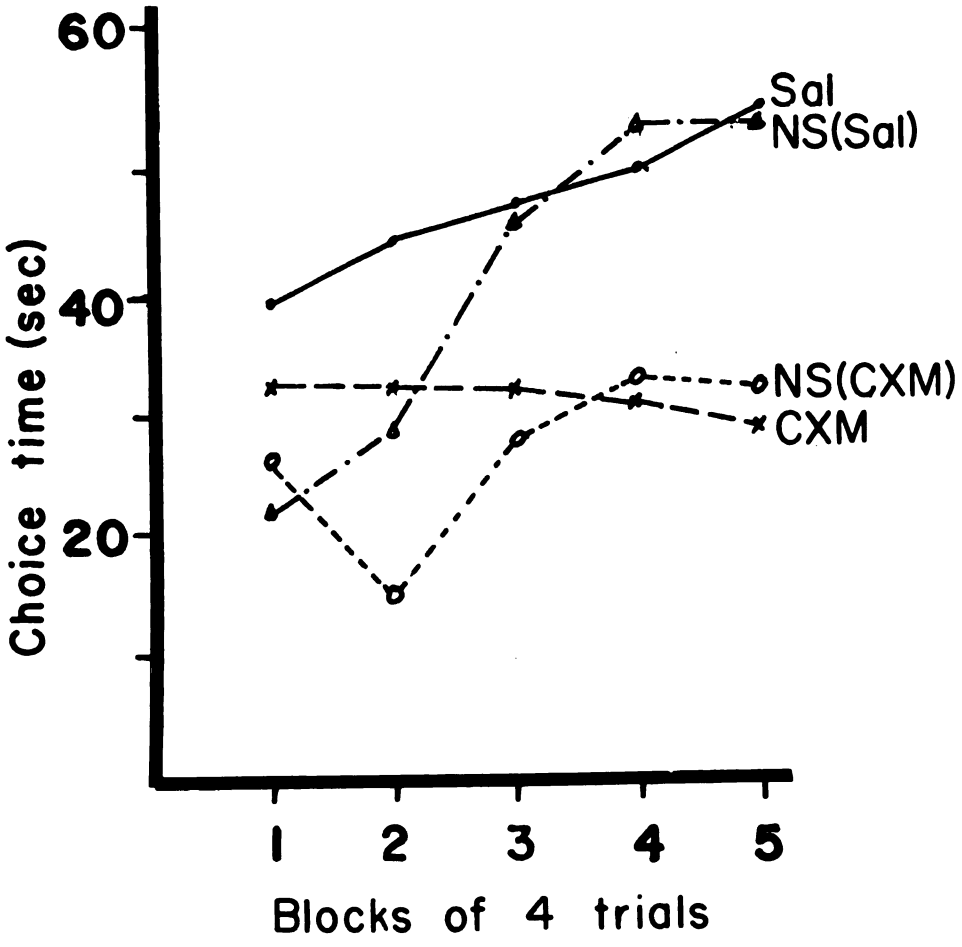


Figure 17. Choice time (time taken to enter the goal box after reaching the choice point) during training for saline-injected and CXM-injected cockroaches given training or T-maze exposure with no shock. The groups are the same as for Figure 16. There is no significant difference between the mean of trials 1-4 for CXM-injected and saline-injected trained animals, but there is a highly significant difference ($p < 0.0001$, two-tailed Mann-Whitney U test) between the means of trials 17-20 for CXM and saline groups.

In any case, animals given CXM reach the goal box faster than control animals, especially in the latter part of the training period. Since similar trends are shown by shock and no shock groups within each drug condition, the change in activity appears to be the result of drug effects only and not an interaction of the drug with shock. A similar conclusion was reached when changes in runway time were considered.

Cycloheximide Treatment in Cockroaches Trained to a Criterion of 5 Correct/6 Trials

Experiments with vertebrates have shown that overtraining animals can prevent the appearance of CXM-induced amnesia. In order to investigate whether the absence of amnesia seen in CXM-injected cockroaches given 20 trials was a result of overtraining, the effect of CXM was tested on cockroaches given fewer training trials. Saline-injected and CXM-injected cockroaches were trained to a criterion of 5/6 correct and tested 24 hours later. The previous experiment showed that cockroaches reach a criterion of 5/6 correct in about 12 trials and thus training to a 5/6 correct criterion would result in substantially fewer than 20 trials.

Methods

The same training procedures were used in this experiment as were used in the previous experiment, where animals were given 20 training trials, except that in the present experiment, cockroaches were trained until they reached a criterion of 5/6 correct turns in the T-maze. A maximum of 20 trials was given. Retention was tested 24 hours after training by retraining animals to the criterion of 5/6 correct. Again a maximum of 20 trials was given.

Results and Discussion

Table 5 shows the mean number of trials required to reach the 5/6 criterion during the training period and during a testing period 24 hours later. There was no significant difference between

Table 5. Mean number of trials to reach a criterion of 5/6 correct turns during training and during testing 24 hours later for cockroaches given either CXM or saline.

Substance Injected	Number of Animals	Trials to 5/6 Criterion Training	Testing
Saline	10	11.1	11.3
CXM	12	13.2	11.3

CXM-injected and saline-injected animals during either training or testing. However, in this experiment, the animals appeared to have no memory of the training experience after 24 hours. Apparently the number of trials received (an average of about 12 trials) was not sufficient to produce retention after 24 hours, whereas retention was observed 22 hours after cockroaches received 20 trials in the previous experiment (Figure 15). In view of the absence of retention shown by both groups of animals trained to the 5/6 criterion, this experiment does not determine if overtraining prevented the production of amnesia by CXM in animals given 20 training trials.

IV. EXPERIMENTS INVESTIGATING PERIPHERAL EFFECTS OF CYCLOHEXIMIDE

Experiments described above indicate that injection of CXM produces impairment of leg lift acquisition in headless cockroaches. The

drug could be acting through a number of different mechanisms, for example, (a) by changing the threshold or activity of peripheral sensory nerves; (b) by altering the motor output of peripheral nerves or altering characteristics of the neuromuscular junction to change the motor response of the leg; (c) by altering the processing of input by interneurons within the ganglion.

Two types of preliminary experiments were done to look at these possibilities. The first experiment investigated the twitching threshold of the leg. When shock is given to the leg during leg lift training, the leg twitches at the femoral-tibial joint (see Figure 18). Experiments were done to see if the lowest shock level which causes twitching (called the twitching threshold) was affected by injections of CXM. This type of experiment involves all three mechanisms mentioned above, but it would determine if CXM produces a gross effect on a reflex response of the leg.

The second type of experiment involved electrophysiological recording from peripheral nerves in an attempt to detect possible effects of CXM on sensory or motor nerve activity. Figure 18 shows the innervation of the leg by nerves 5 and 6, the two nerves recorded from in these experiments. Nerve 5 is the only nerve which innervates the tibia and tarsus and thus would be the only source of sensory input from this portion of the leg. Nerve 5 is involved mainly in leg extension and nerve 6 innervates flexor muscles and is probably involved in the leg flexion response to shock. Thus, a change in activity induced in these nerves by CXM could affect leg lift acquisition. The electrophysiological experiments described below

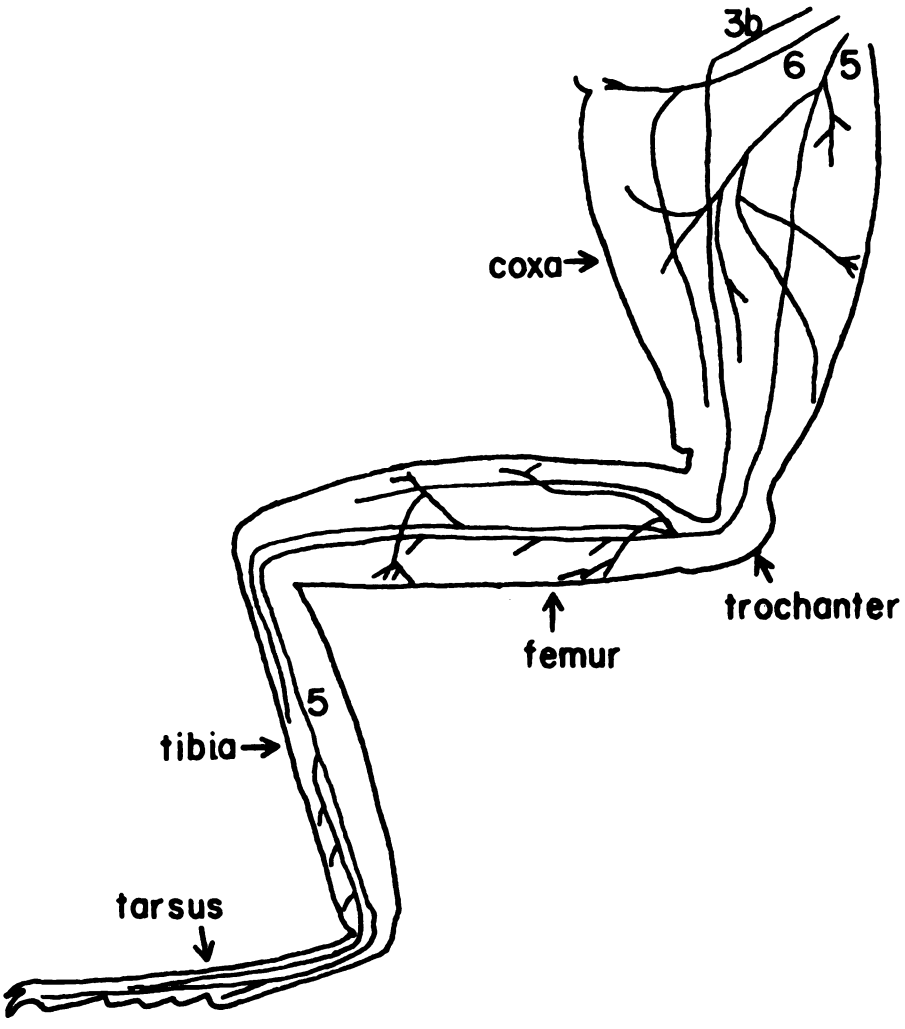


Figure 18. Innervation of the mesothoracic leg by nerves 3b, 5 and 6. The innervation of the prothoracic leg is very similar. Figure taken from Guthrie and Tindall (1968).

involve comparing the activity of nerves 5 and 6 in response to electrical or mechanical stimulation of the leg in CXM-treated and control animals.

Twitching Threshold

Methods

In order to examine possible effects of CXM on the sensitivity of the leg to shock, headless animals were injected and wired by the same method as for leg lift training. The amount of current passed through the leg was increased from a very low level until the leg started twitching and then the current was decreased. The magnitude of shock, recorded as voltage levels, was recorded at four points during the increase and decrease in shock level. During the increase in voltage, the initiation of tarsus twitching and the point at which the tibia also started to twitch were noted. As the shock level was decreased, the points at which the tibia stopped twitching and at which the tarsus stopped twitching were recorded.

Results

Figure 19 shows the results of these experiments. There was no difference between control animals and animals given 37.5 µg of CXM--the dose level at which the leg lift training was conducted. In addition, a dose of 75 µg was given to six animals. The threshold level appeared to decrease, but the difference between the 75 µg and control groups are not highly significant. The differences at points B and C are significant at the 0.10 level (two-tailed Mann-Whitney U test); the other differences are not significant at this level. Thus CXM at high doses may cause a decrease in twitching threshold level. Drug-induced changes in the twitching threshold have been observed in

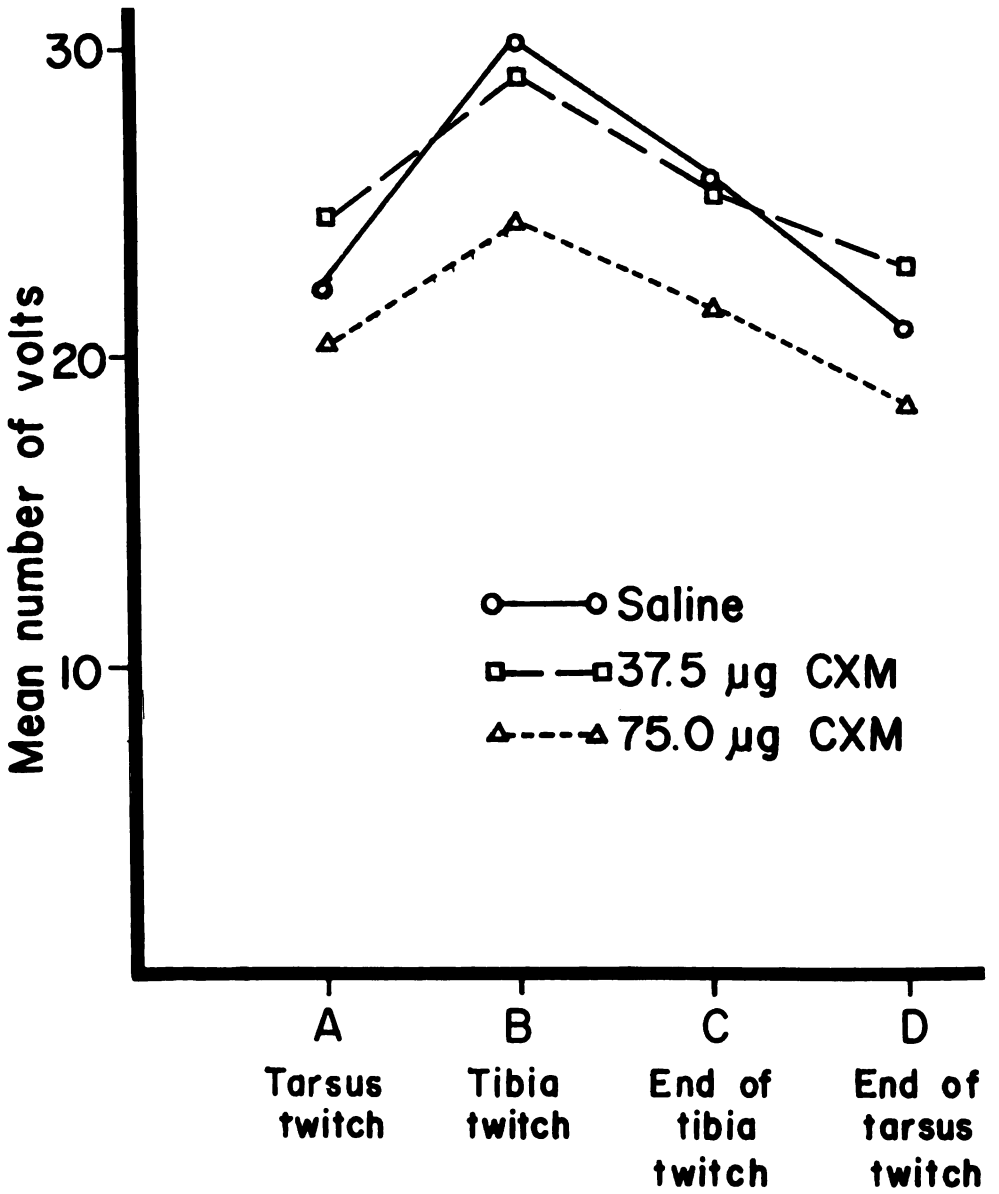


Figure 19. Magnitude of shock at which twitching of the tarsus and tibia was initiated or terminated. These measures are an indication of the twitching threshold of the cockroach leg after injection of saline or CXM. There were 10 animals in the saline and 37.5 ug groups and 6 animals in the 75.0 ug group. There is no significant difference between control animals and animals injected with 37.5 ug CXM at any point. The differences between the control group and the group given 75 ug CXM are significant at the 0.10 level (two-tailed Mann-Whitney U test) for points B and C; the other differences are not significant at this level.

cockroaches. Eisenstein (personal communication) reported a decrease in twitching threshold of the leg after injection of actinomycin D, another antibiotic. Although a decrease in twitching threshold is not seen at the 37.5 μg dose of CXM which produces behavioral changes in headless cockroaches, the changes seen at the higher dose of CXM and with actinomycin D may be indicative of subtle changes which occur peripherally at low doses and which can influence leg behavior under appropriate conditions. However, the influence of any such effects on leg behavior during leg lift training remain to be demonstrated.

Electrophysiological Recording

Methods

Two main sets of experiments were performed to investigate the effects of CXM on peripheral nerve activity. In one set of experiments (Experiment A) animals were injected with either CXM solution or saline solution and the activity of nerves 5 and 6 in response to mechanical stimulation of the leg was recorded. In the second set of experiments (Experiment B) one animal was used as its own control. Nerve 5 was covered first with saline and then with CXM solution (12.5 mg/ml). During this process the activity of nerve 5 in response to electrical stimulation was recorded at several intervals. In both sets of experiments, the parameters of nerve activity measured included threshold and duration of spike burst, and in some cases, latency of onset of the response. The threshold was measured as the lowest stimulating voltage at which a spike burst response was seen on the oscilloscope or heard from the speaker. Each of these methods will be described in more detail below.

Experiment A--Mechanical Stimulation. In these experiments, some of the details of the preparation of animals was varied throughout the course of the experiments. Basically, all animals were prepared for recording with hook electrodes from nerves 5 and 6 of the prothoracic segment. The animal was put under carbon dioxide anesthesia. After decapitation, the prothoracic ganglion and nerves 5 and 6 were exposed; nerve 4 was cut to prevent interference. Mechanical stimulation involved waxing a pin, which was attached to a loudspeaker, to the tibia near the femoral-tibial joint and in line with the bending of the leg at the joint. When the speaker was stimulated, the pin moved forward, bending the leg at the joint a constant amount (see Figure 20). Difficulties in placing the pin in identical locations on different animals would allow for some variation in stimulus intensity. Stimulation was given at a frequency of 1/sec at various intensities. The circuitry for stimulation and recording is shown in Figure 20. Other aspects of the procedure, including the method by which the gut was removed and the type of electrode (monopolar or bipolar), were varied among animals.

Experiment B--Electrical Stimulation. Each animal was placed under carbon dioxide anesthesia during preparation. The head was removed, and the posterior end of the animal was elevated so the gut fluids could drain through the neck. The prothoracic ganglion and nerve 5 were exposed and the nerve was cut for suction electrode recording of the distal end. A well was made out of clay so the nerve could be flooded with solution. Wires (0.002" diameter) were placed in the leg for shock delivery. One wire was placed into the tissues of the tibia after removal of the tarsus; the second wire was

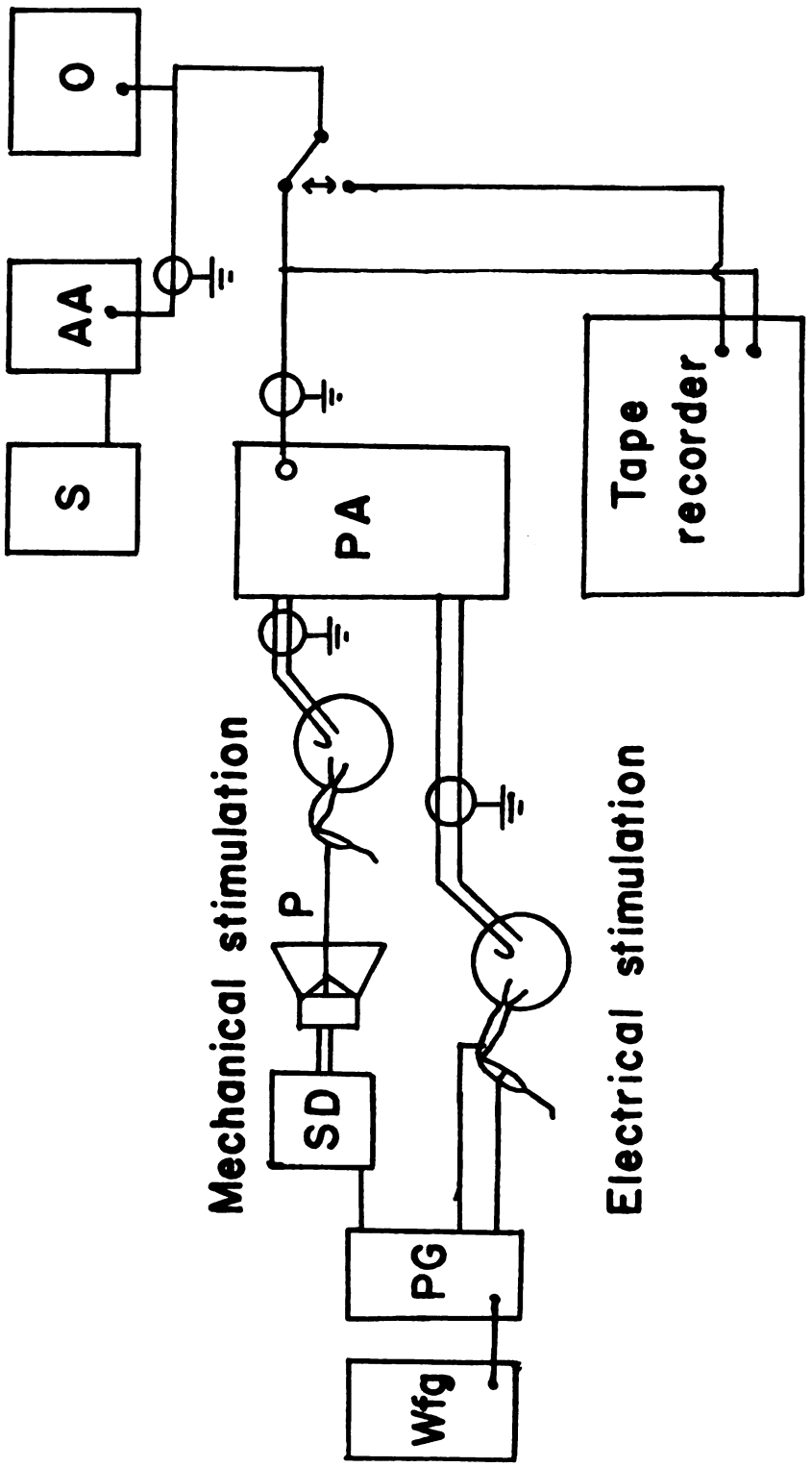


Figure 20. Circuitry for mechanical and electrical stimulation of the leg and electrophysiological recording from nerves 5 and 6 after administration of CXM or saline. See text for details of procedure. The electrical activity was monitored both with an oscilloscope and with a loudspeaker. Abbreviations used: Wfg, Tektronics 162 wave form generator; PG, Tektronics 161 pulse generator; SD, speaker driver; P, pin attached to speaker; PA, Tektronics 122 pre-amplifier; S, speaker; AA, audio amplifier; O, Tektronics 502 oscilloscope.

put into the femur through a hole in the cuticle. Both wires were waxed into place. The circuitry for stimulation and recording is shown in Figure 20. Pulses were delivered at a frequency of 1/800 msec at various amplitudes.

Results

Experiment A--Mechanical Stimulation. The electrical response to bending of the leg at the femoral-tibial joint consisted of a burst of impulses lasting 15-30 msec. Figure 21a shows a typical response. Table 5 gives the results of threshold and duration measurements in response to mechanical stimulation for animals injected with CXM or saline. For most animals, several readings were taken of the threshold level. Duration was measured only in some of the animals. A viable response was obtained from nerve 6 in fewer animals than from nerve 5, probably because nerve 6 was smaller and harder to record from. At the beginning of the recording from animal no. 14, a new speaker was used, changing to some degree the characteristics of the stimulation. There is no significant difference between the CXM and saline injected animals for any measure, although the variation among animals is large, which could mask small differences. The range of response is also similar for both groups.

Experiment B--Electrical Stimulation. In an attempt to minimize the variation which complicates a comparison of CXM and saline treated animals and to use the same stimulus (shock) as used in the behavioral experiments, animals were prepared according to the methods for Experiment B.

An example of the burst of impulses produced by electrical stimulation is shown in Figure 21c. This should be compared to

Table 6. Threshold and duration measurements of evoked electrical activity of nerves 5 and 6 in response to bending the leg at the femoral-tibial joint.

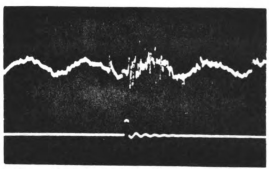
Substance Injected	Animal Number	Nerve 5 Threshold (mV)	Nerve 5 Mean of Threshold Measures	Nerve 5 Duration (msec)	Nerve 6 Threshold (mV)	Nerve 6 Mean of Threshold Measures
CXM	5	100,200	150	-	-	-
CXM	7	100,75,20	65	25	450	450
CXM	11	500,550,300	450	20	175,200	177
CXM	15	40,50,25,50	41	15	200,175,150	175
Saline	6	200,200	200	25	400,550,500	483
Saline	10	200	200	-	-	-
Saline	12	400,200,100	350	30	100,100	100
Saline	14	20,10,10,20	15	-	-	-
Saline	16	175,150,150	158	15	-	-
Mean of CXM Animals			176	20	-	267
Mean of Saline Animals			185	23.3	-	291

Figure 21b, which shows only the stimulus artifact produced by a sub-threshold pulse. Since the stimulus artifact was so large, the gain had to be reduced; thus the spikes occurring on the tail of the artifact appear smaller than for mechanical stimulation. The threshold of response was determined at various times before and after the application of CXM solution to the region surrounding nerve 5. Figure 22 shows the response threshold of nerve 5 as a function of time for individual animals. There is no indication that exposure of the nerve to CXM changes its threshold to electrical stimulation.

In one animal, the duration of the burst, the number of spikes in the burst, and the latency (time between stimulus and onset of the burst) were measured when the nerve was bathed with saline followed by CXM solution. There was no consistent change in any of these measures after CXM was applied.

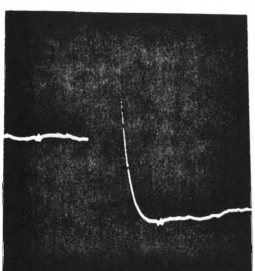
In these experiments the nerves deteriorated about 30 minutes after being flooded with CXM solution. After this time the response was either very small or not observed. The nerves in these experiments were exposed to a much greater amount of CXM than nerves in the behavioral experiments or in the mechanical stimulation electrophysiological experiments (Experiment A). The nerves in Experiment A remained viable for at least 1 or 2 hours after injection of CXM into the animal. Thus the very high levels of CXM may have caused deterioration of the nerves in Experiment B.

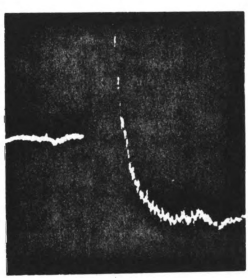
In summary, the electrophysiological recording performed in Experiments A and B did not detect any changes in activity of nerves 5 or 6 due to CXM administration. Thus there does not appear to be a gross effect of CXM on evoked activity in peripheral nerves.



21a

10 msec





21b

21c

10 msec

Figure 21. Examples of evoked electrical activity recorded from nerve 5. a: evoked response to bending of the leg at the femoral-tibial joint in Experiment A; upper trace, spike burst response; lower trace, deflection shows onset of stimulus; settings: 0.1 V/cm. b: stimulus artifact accompanying electrical stimulation of the leg in Experiment B. c: evoked burst of impulses occurring on the tail of the electrical stimulation artifact seen in b; settings: 0.5 V/cm.

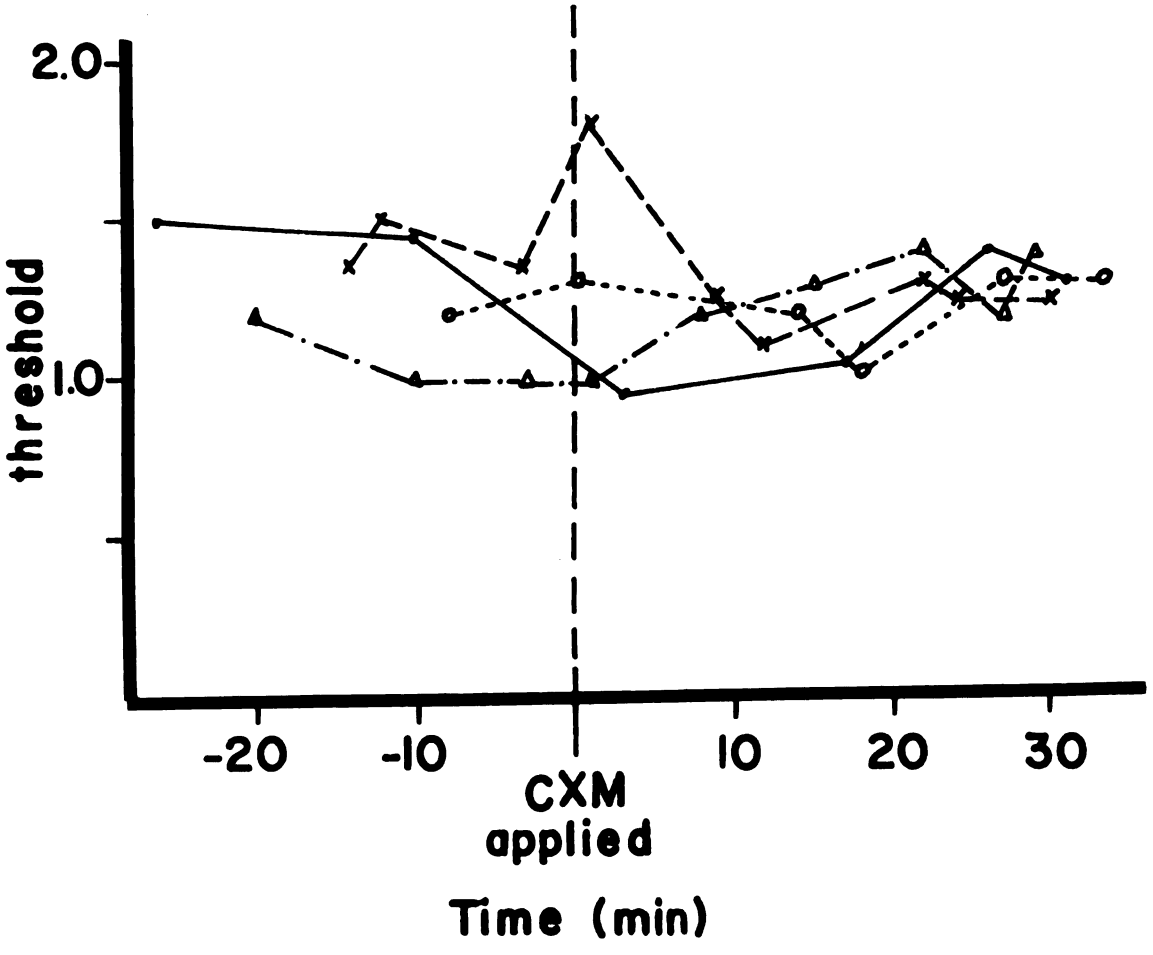


Figure 22. Response threshold of nerve 5 in individual animals as a function of time before and after application of CXM solution to the nerve. The threshold was measured as the lowest intensity of shock applied to the leg which evoked a burst of impulses in nerve 5. There was no difference in response threshold as a result of CXM application.

Discussion

The above experiments were intended as preliminary work to try to detect CXM-induced changes in peripheral electrical activity which might play a role in the CXM-induced impairment of leg lift acquisition in headless cockroaches. An effect of CXM on the peripheral nervous system in the cockroach could be localized at a number of different sites including: 1) response of sensory fibers to input; 2) activity of efferent fibers; and 3) characteristics of the neuromuscular junction. The experiments described above investigate the effects of CXM on a) the response of sensory fibers to electrical stimulation by recording from the distal end of cut nerve 5 (Experiment B), and b) the motor output (which could include changes in sensory response, central processing or efferent activity) by recording from intact nerves 5 and 6 (Experiment A). No differences between control and CXM-treated preparations were seen in these experiments, thus indicating on a gross level no evidence of peripheral CXM effects, and raising the possibility that the CXM effects are ganglionic. However, there was a great deal of variability in the characteristics of the evoked potentials in each nerve and among different nerves, which would tend to mask the detection of any small differences. Since the experiments reported above would only reveal changes involving simple facilitation or inhibition of responses, it is possible that the effects of CXM are contingency-related; i.e. they only emerge if shocks are administered under certain conditions--for example, when the firing rate of a neuron is above or below a given level, rather than when shocks are given randomly with respect to the activity of a neuron. If this occurred, then the procedure used in these experiments would not have

detected it. This type of effect would be consistent with the behavioral results, in which CXM caused an increase in initial leg activity of P animals (where the shock stimulus was contingent on leg extension) but did not produce an increase in leg activity of R animals (where the shock stimulus was administered randomly with respect to leg position).

Further Experiments to Detect Peripheral Changes. The location and nature of possible peripheral changes underlying the CXM-induced acquisition impairment in headless animals could be investigated by a number of electrophysiological experiments incorporating the possibility of contingency-related changes, including the following examples.

1) Response of individual sensory fibers to stimulation of one receptor could be measured for control and CXM-treated preparations to determine if CXM affects the response characteristics of sensory fibers on the cellular level.

2) While it is not possible to separate a CXM-induced alteration of ganglionic information processing from an alteration of motoneuron activity without recording from relevant interneurons within the ganglion--a difficult undertaking, recording from peripheral motoneurons should determine if either of these sites (central processing or motoneuron activity) is affected by CXM. Further recording from nerve 6 and recording from nerve 3b, both of which innervate flexor muscles, could reveal changes in electrical activity produced by CXM at the level of the ganglion or efferent fibers. Recording should be done of a) spontaneous activity; b) evoked response to regular stimulation of the leg at regular intervals; and c) evoked response to stimulation contingent on the rate of discharge of the units being

monitored (an electrophysiological analogue to leg lift training). This could determine if CXM can produce efferent activity changes and if the changes are contingency related.

3) An investigation of the CXM effects at the level of the neuromuscular junction could be pursued by recording muscle tension in appropriate flexor and extensor muscles while monitoring spike activity in the motor neurons innervating those muscles. This should be done for the same three conditions described above in 2).

DISCUSSION

The basic question investigated in this dissertation concerned the nature of the effects of CXM on learning and retention in cockroaches. The observation that CXM impairs the rate of acquisition of leg lift training in headless cockroaches was replicated. The research reported here demonstrated that the CXM-induced acquisition impairment effect is specific to headless cockroaches--CXM did not impair acquisition of leg lift learning or T-maze learning for intact cockroaches. This suggests that there is something unique about the headless preparation which makes it sensitive to CXM effects during training. The question of the nature of CXM effects on headless cockroaches will be discussed in the first part of this discussion section.

A second question that was explored concerned the production of amnesia in cockroaches by injections of CXM given before training. No memory deficits were produced by CXM in the T-maze experiments, while in similar experiments on vertebrates, severe memory deficits have been produced by CXM and other antibiotics. The second part of this section will discuss possible explanations for the absence of effects of CXM on retention in cockroaches.

When both intact and headless cockroaches were given leg lift training to compare the effects of CXM on learning in the two preparations, it became possible to compare a number of characteristics

of learning between headless and intact preparations trained under comparable conditions. Such a comparison is important in determining whether the headless cockroach can be reliably used as a model system to investigate characteristics and mechanisms of learning which would be presumed to occur in the intact animal. Differences in behavioral characteristics between headless and intact preparations will be discussed in the third part of this discussion section.

Nature of the Cycloheximide Impairment Effect in Headless Cockroaches

The mechanism of the CXM impairment of leg lift acquisition in headless insects, i.e. whether CXM affects acquisition through changes in activity levels or whether CXM impairs the association process underlying learning, has been a major question in this area.

Since CXM does not impair learning in intact animals, the mechanisms responsible for the CXM-induced impairment in headless animals are specific to the headless preparation, and this discussion will be limited to the nature of the impairment effect in the ventral nerve cord. Two theories can be proposed to explain the observed effects of CXM: (1) CXM could impair the learning process which operates in headless animals, or (2) CXM could alter initial levels of activity of the leg, making it more difficult for the animal to perform the leg lift task. Each of these possibilities, and the evidence related to each, will be discussed in more detail below; physiological aspects of the headless preparation which could be important in understanding the nature and specificity of the CXM effect will be described; and further experiments will be proposed to test the validity of the two theories.

The hypothesis that CXM impairs the learning process in headless cockroaches assumes that CXM inhibits the association process between shock and leg extension occurring in the ganglion, rather than having peripheral effects on the leg. Several aspects of the research reported here are consistent with this theory: (a) CXM-injected P animals take longer during leg lift learning to reach an asymptote than saline-injected P animals. (b) Careful analysis of the behavior of R animals given CXM or saline (Figures 4,5) did not reveal a significant difference in leg activity or leg position. A description of R leg behavior, an important control for CXM activity effects, was not reported in previous work investigating effects of CXM in leg lift learning. The absence of an effect of CXM on the number of dips made by the R leg (leg activity) during a period when CXM increases the number of dips made by the P leg (Figure 5) argues against a hypothesis that CXM has a major effect on activity of the leg. (c) There was no change in the twitching threshold of the leg after administration of the 37.5 μg dose of CXM used in the leg lift experiments, suggesting that there is no effect of this dose of CXM on sensitivity of the leg to shock. (d) No difference in evoked electrical activity was observed between CXM and saline treated preparations during preliminary electrophysiological experiments, indicating on a gross level no evidence of peripheral CXM effects on electrical activity.

However, other aspects of the leg lift experiments support a hypothesis that CXM affects activity levels rather than actual learning. This hypothesis would include all perhipheral effects of CXM, including changes in sensory and motor activity, which might affect leg behavior during leg lift training. Results which are consistent with this

hypothesis include the following. (a) The initial activity of the headless CXM-injected P animals (see Figure 5) was much higher than for saline-injected P animals during the first 10 minutes of training, which corresponds to the period of acquisition impairment in Figure 3. (b) If CXM slowed down the rate of learning, the slope of the learning curve of the CXM group should be less steep than for the saline group. As shown in Figure 3, the CXM group starts at a much higher level, and while it is difficult to determine an exact slope for the initial part of the curve, the slope is not obviously different from the slope for the saline-injected group. This data suggests that CXM may affect only the starting levels and not the rate of learning of headless animals. (c) An experiment by Glassman et al. (1970) (see page 27) suggests that the effect of CXM is on performance of the leg lift task rather than on the rate of learning. (d) The previously published reports claiming an inhibitory effect of CXM on learning (Brown and Noble, 1967; Kerkut et al., 1970) do not present data in a suitable form or contain the proper controls to evaluate whether learning per se is influenced. For example, Kerkut et al. show a learning curve for only one animal given CXM and present the remainder of the data in the form of either number of shocks to criterion, or time to criterion, and neither of these parameters effectively distinguishes the effect of the drug on the rate of learning from the effect on initial activity levels. (e) Although the 37.5 μg dose of CXM did not affect the twitching threshold of the leg, there did appear to be some decrease in threshold after administration of a larger dose suggesting the possibility of some type of CXM effect on the responsiveness to shock. Eisenstein (personal communication) demonstrated a decrease in the twitching threshold of

the prothoracic leg of cockroaches after administration of actinomycin D, an antibiotic that inhibits RNA synthesis. The possibility that CXM may cause a change in the threshold of response to a stimulus is supported by observation of the behavior of cockroaches in the T-maze, where the locomotor response to gentle prodding was greatly enhanced in CXM-treated animals. Thus it is possible that a change in the response of the leg to shock exists, but did not appear as a change in the twitching threshold at the 37.5 μg dose of CXM.

In view of the somewhat conflicting evidence, the nature of the CXM impairment effect is not yet resolved. CXM does not appear to affect the innate activity levels shown by the R animals, but the drug somehow makes maintained leg flexion more difficult to achieve for P animals. It is not immediately clear why only headless animals should be affected in this way by CXM. Perhaps decapitation produces changes in the preparation that make it susceptible to the actions of CXM in a unique way. In order to investigate this possibility and get a better understanding of the characteristics of the preparation with which we are dealing, the author will describe physiological changes which have been reported to occur after decapitation.

In the insect nervous system there is local control by thoracic ganglia of locomotion and other motor activity which can occur in the absence of the head ganglia. In general, central regulation of these local systems is accomplished largely through inhibition from the brain. For example, in the cockroach, the control of efferent fiber activity by inhibitory fibers descending from the brain has been demonstrated for thoracic motoneurons by Pringle (1940). Pringle showed that decapitation

produced hyperactivity of all slow fibers to leg muscles, but especially to depressors (extensors). Lack of coordination was observed in the levator (flexor) responses and the preparation was usually very responsive to air currents. Roeder et al. (1960) found that the increase in efferent activity produced by decapitation usually took the form of regular volleys rather than the raising of a steady frequency, the frequency of the volleys varying in different fibers of a nerve.

Thus there are makred changes in the electrical activity of peripheral nerves as a result of decapitation. Similar changes can be produced chemically, since Milburn et al. (1960) found that extracts of the corpus cardiacum (an endocrine gland) administered to intact cockroaches produced heightened activity in thoracic efferents. This was interpreted as blocking of inhibition from the head.

On the cellular level, it has been observed that isolation of a ganglion in invertebrates can produce marked changes in certain synaptic interactions. For example, Jansen et al. (1974) found a change from excitation to inhibition at identified synapses in leech ganglia isolated by severing all incoming and outgoing fibers. Since the synaptic changes observed in the leech ganglion occurred over a period of weeks after the lesion, the mechanisms responsible for these changes may be completely different from mechanisms producing heightened efferent activity after cockroach nervous system lesion. In any case, lesion experiments on both the leech and the cockroach suggest that either intrinsic activity of neurons or synaptic interactions among neurons, or both, may be substantially altered after lesions of the nervous system, and such changes would be expected to produce behavioral alterations in the animals.

Since the headless animal displays a markedly different level of efferent nerve activity and has different information processing characteristics due to the absence of the brain (see page 111 for further discussion of this point) than the intact animal, it is not too surprising that CXM could affect the headless preparation in a unique way. This could be done either by impairing the learning which operates in the ventral nerve cord or by altering the increased nerve activity levels which occur after decapitation. Two types of experiments will be described which would test these possibilities.

(1) It is possible that CXM acts to impair the ability of the P leg to maintain a flexed position. Since R animals flex very little but instead maintain their legs in an extended position most of the time, a CXM-induced effect which makes leg flexion more difficult might not be observed in R animals. This possibility could be tested by training headless animals to extend a leg to avoid shock. If CXM-injected animals learn as well as saline-injected animals to extend to avoid shock, then the effect of CXM is not on the learning process, and must be on some aspect of performance of the leg lift task. An impairment of leg extension acquisition by CXM would provide good evidence for an effect of CXM on the learning process.

(2) The nature of the CXM impairment effect can also be investigated by electrophysiological recording in the ventral nerve cord in which the electrical activity of flexor and extensor motoneurons is recorded after CXM of saline administration under conditions or non-contingent stimulation and contingent stimulation. The non-contingent stimulation would consist of applying shock input at regular intervals or random intervals and monitoring the spike activity of peripheral nerves

(similar to Experiment B of the electrophysiological recording experiments). The contingent stimulation would consist of applying shock input contingent upon the rate of discharge of a neuron, as in Hoyle's experiments. The experiments by Hoyle (1965) suggested that the discharge rate of a neuron can be conditioned to increase or decrease if contingent shock stimulation is given to the leg when the discharge rate falls below or rises above a "demand level". This is an electrophysiological analogue of leg position learning, but its reliability remains to be determined. Assuming that this electrophysiological analogue is shown to be a reliable indicator of learning, the following conclusions could be drawn from the proposed experiments. If CXM has no effect, over a period of time, on spontaneous activity or on evoked activity under conditions of random or regular stimulation, but CXM prolongs the time required to condition the rate of discharge of a motoneuron through contingent stimulation, then it may be concluded that CXM affects learning in this preparation. If CXM does not affect the conditioning process, then it would appear that CXM does not affect learning, but impairs some aspect of leg lift performance.

These electrophysiological experiments, together with the experiments investigating effects of CXM on leg extension learning discussed above, should determine if CXM produces its acquisition impairment in headless cockroaches by altering the ability of the leg to perform the leg lift task or by interfering with the ganglionic association process underlying learning.

Effects of Cycloheximide on Retention

The T-maze experiments reported above indicated that CXM had no observable effects on retention in cockroaches at intervals up to 22 hours

after training. Kerkut et al. (1970) also reported no effect of CXM on retention of headless cockroaches trained in the leg lift paradigm. These results are in marked contrast to the effects of CXM observed in vertebrates, where a similar amount of protein synthesis inhibition produced by CXM induced severe impairment of memory beginning 3-6 hours after training. There are three explanations for this discrepancy between insects and vertebrates.

(1) CXM may impair retention in cockroaches under certain conditions, but the proper parameters of training have not yet been encountered. This possibility can only be tested by continuing to observe the effects of CXM using various types and parameters of training.

(2) CXM could have different physiological effects in different types of animals, such that in vertebrates memory consolidation processes are impaired while in cockroaches the same processes are unaffected by CXM. Although possible, this seems unlikely in view of the similarity of cellular function of nerve cells throughout the phylogenetic kingdom and the demonstrated common inhibition of protein synthesis by CXM in cockroaches, goldfish and mice.

(3) The physiological basis of memory consolidation may be different such that in insects it is not susceptible to the actions of CXM. To consider further a possible molecular basis of this alternative, proposed mechanisms of protein synthesis inhibitors on memory and their applicability to the cockroach system will be discussed.

Barraco and Stettner (1975) proposed a model to explain the diverse effects of antibiotics on memory. In this model the cholinergic system mediates information storage (memory) while the adrenergic

system mediates information retrieval and integration (learning). The adrenergic system would exert its effect by leading to facilitatory changes (during training) in the specific network of synapses that are associated with the training system and causing preferential reactivation (during testing) of this previously facilitated network (Serota et al., 1972). The adrenergic changes would lead to permanent alterations of the cholinergic networks, establishing the memory trace. In the proposed model, the neurochemical system interacts with a "continuum of preparedness", defined as a system in which organisms are prepared to associate certain events, unprepared for some, and contraprepared for others (Seligman, 1970). For example, animals are highly "prepared" (and thus learn easily) to associate a defensive action, such as escape, with shock avoidance, while they are unprepared (and have a difficult time learning) to associate an appetitive response with shock avoidance. Thus, the adrenergic activational mode would participate less in the acquisition of highly "prepared" tasks, in which the stimulus-response associations are highly "primed" within the species-specific repertory of the organism, and participate more in the unprepared tasks in which the stimulus-response associations are not included in the species-specific behavioral repertory. Superimposed on the preparedness continuum would be the level of training; overtraining produces, in effect, highly prepared association as a result of repetition. Consequently, the predicted effect of an antibiotic on memory would depend upon the interaction of several factors: the pharmacological effects of the antibiotic used and its time of injection, the level of adrenergic activation required for stimulus-response associations (degree of preparedness) and the level of training.

According to Barraco and Stettner, results of experiments on vertebrates imply three separate pharmacological effects of antibiotics on memory: (1) the effect on retrieval of puromycin injected long after training (possibly mediated by peptidyl-puromycin fragments); (2) the effect of glutarimides, injected before or immediately after training, on activated task-specific networks of adrenergic neurons (mediated by selective interference with the adrenergic neurotransmitter process and reducing the "strength" of consolidation); (3) the effect of puromycin injected before or immediately after training directly on a task-specific network of cholinergic neurons which are involved in consolidation of the engram (mediated by selective interference with the cholinergic neurotransmitter process).

There is some evidence to support this type of model in mammals, but all aspects of such a model may not be applicable to insects. Both acetylcholine (ACh) and norepinephrine (NE) have been demonstrated in the brains of cockroaches (Treherne, 1966; Frontali, 1968; Frontali and Haggendal, 1969) and both have been proposed as synaptic transmitters (see Gerschenfeld, 1973), but the role of these putative transmitters in influencing insect behavior has received little attention. Maroli and Bettini (1975) recently investigated the effects of amphetamine (a stimulant which may act in part by increasing the concentration of NE at noradrenergic postsynaptic receptors) on the cockroach. Maroli and Bettini found that doses of amphetamine lower than the LD_{50} value (525 mg/kg) produced an increase in locomotor activity and a state of hyperexcitability. Doses higher than the LD_{50} value caused a decrease in locomotor activity. A study on ants (Kostowski, et al., 1975) reported that catecholamines are involved in

the organization of aggressive behavior in ants. Thus, catecholamines are important in the insect nervous system, but further research must be done to determine if biogenic amines play analogous roles in the organization of insect and mammalian behaviors, especially in arousal and learning behaviors.

The "continuum of preparedness" concept also has not been investigated in insects, but it is possible that much of the learning of which insects are capable consists of species-specific, highly prepared stimulus-response associations. If this is true, then the adrenergic system may be involved to a lesser degree, or not at all, in the consolidation of memory in insects. Thus, if CXM exerts its memory impairment effects in vertebrates through inhibition of the adrenergic activating system, and if this system is not a principle component of memory consolidation in cockroaches, then CXM would not be expected to affect retention in cockroaches. However puromycin would be expected to impair memory in cockroaches if Barraco and Stettner's theory on the effect of puromycin is correct and if memory is stored through the cholinergic system in cockroaches. Since research investigating the effects of puromycin on retention in cockroaches has not been conducted, the applicability of this model to the insect nervous system, in terms of the proposed puromycin effect directly on the cholinergic memory storage system, cannot be evaluated. Further experiments should be done investigating the effects of both puromycin and CXM on learning and retention in cockroaches to determine if memory deficits can be produced, and if so, under what conditions and by which drugs. Such experiments are necessary in order to draw any firm conclusions concerning the similarity of learning and memory processes on the molecular level throughout phylogenesis.

Comparison of Learning in Headless and Intact Animals

The above experiments demonstrated, and previous experiments have reported, that intact cockroaches trained in the leg lift paradigm have a slower rate of acquisition than headless animals (Pritchatt, 1968; Kerkut et al., 1970). The main explanation of this paradoxical result has been that the sensory input received by the head interfered with the association between leg position and shock; i.e. the intact animal was "distracted" by the additional sensory input. This theory could account for some dissimilarity between headless and intact preparations in behavior characteristics, e.g. rate of acquisition, but would not seem to explain all differences between headless and intact animals in activity levels, dip durations and changes in rate of acquisition after CXM administration.

The behavioral characteristics which have been observed in the present studies suggest that there may be a fundamental difference in the learning characteristics of headless and intact animals. Such differences are difficult to detect in most of the experiments which have been reported because the intactness of the nervous system has not been considered an important variable, and characteristics of learning have been compared without regard for the type of preparation used. Different experimenters have trained different legs, and used different preparative techniques and different parameters of shock, all of which may affect the rate of learning or other characteristics of the behavior. In the experiments reported in this dissertation, comparable procedures were used for headless and intact animals and a number of

differences in learning characteristics emerged. These will be summarized and a theory will be proposed which could provide a basis for such differences.

Differences in leg lift learning characteristics between headless and intact preparations include the following:

(a) CXM impairs acquisition in headless animals, but the drug shows no evidence of acquisition impairment in intact animals.

(b) Comparison of learning curves of the two saline-injected preparations indicates that intact cockroaches display a slower rate of acquisition than headless animals (see Figures 3 and 8). This observation confirms previous results (Pritchatt, 1968; Kerkut, et al. 1970). The shape of the learning curve for intact saline-injected animals suggests the possibility of a "backsliding" tendency, i.e. a period of regression following initial improvement. Although this cannot be confirmed statistically, backsliding phenomena have also been observed in the intact cockroach (but not in the ventral nerve cord preparation) given leg lift training by Kerkut, et al. (1970) and in intact insects given other types of training (see Guthrie and Tindall, 1968).

(c) The activity of the two preparations, as measured by the number of dips, was different, with the intact animals more active throughout the training period (see Figures 5 and 9) and especially at the beginning of training.

(d) Analysis of characteristics of escape learning, as determined by a decrease in dip duration during training (Figures 6 and 10), suggests that a component of escape learning is present for intact cockroaches during acquisition of the leg lift task. However, there

is no reliable indication that escape learning plays a part in the learning of headless cockroaches.

(e) The dip duration for both P and R intact animals is much higher than for headless P animals (see Figures 6 and 10), suggesting a difference in the response of the leg to shock. From qualitative observations, the leg of a headless animal appears to flex very quickly when shock is first received, leading to short dip durations even at the beginning of training. In contrast, the leg behavior of intact animals appears to be much more deliberate, and rapid withdrawal of the leg is not as prominent. This suggests that the brain exerts control of leg activity to some extent, and removing the brain releases some inhibition of leg flexion activity. However, the dip duration of headless R animals is much higher than for either P or R intact animals. This could be explained by an interaction between P and R leg behaviors which may be very important in influencing behavior of the R leg. Since the headless P animal initiates relatively few shocks during training, the R animal is shocked relatively few times and spontaneous leg behavior (i.e. extension) seems to predominate over changes in leg behavior which may be produced by shock. Intact P animals initiate many more shocks during training than headless P animals and intact R animals thus receive more shocks than headless R animals. A much larger percentage of the leg behavior of intact R animals may be governed by shock response, leading to more changes in leg position and resulting in shorter dip durations when shock is received during a dip. The dip duration characteristics of the R leg could be determined either by the presence of the head in controlling leg behavior or by the amount of shock received by the leg. These alternatives could be tested by

varying the number of shocks given to the R leg of each preparation, either by changing the level of the solution in which P dips or by artificially applying shocks to R and observing changes in dip durations.

(f) The data obtained during the testing period indicated that the headless R animals initiated more shocks during testing than did headless P animals during training, while the intact R animals initiated fewer shocks during testing than did intact P animals during training. Thus,the "random shock" stimulation received by both headless and intact R animals during training had different effects on future behavior of the two preparations, suggesting a difference in processing of the R experience by the brain and the ventral nerve cord.

The above examples represent substantial differences between headless and intact preparations. Chen, et al. (1970) also found a difference in learning between intact and headless preparations. However, in their experiments, intact animals were much better able to associate a subthreshold shock with the subsequent occurrence of a severe shock than headless animals. The differences seen in learning between intact and headless animals may be partially due to alterations in the intrinsic activity of efferent nerves which occurs after decapitation (see page 103 for further details), but they may also indicate a fundamental difference in the types of learning which can occur in the brain in contrast to the thoracic ganglia.

A theory which could explain such differences can be proposed on the basis of Razran's (1971) classification of leg position learning in the ventral nerve cord as aversive inhibitory conditioning. Razran defined aversive inhibitory conditioning as "progressive decrement of a reaction through association with a reaction that inhibits it without

formation of another reaction". This is a form of learning less complex than either classical or instrumental conditioning and probably evolutionarily more primitive. Aversive inhibitory conditioning can be used to describe leg lift learning in headless cockroaches by assuming that flexion is the predominant unconditioned response to shock and the resting posture involves mainly extension. In the standard P learning situation, extension is punished by shock, thereby inhibiting the extension component and resulting in leg flexion. Since flexion upon receipt of shock appears to be a rapid stereotyped response of headless animals, this hypothesis could easily explain the behavioral changes which occur. However, in the case of intact animals, the flexion response to shock is not as rapid as in headless animals, as shown by the longer dip duration values. Also, the intact animals appear to acquire a new reaction to shock, as shown by the fact that escape learning occurs during training (Figure 10). Thus the learning characteristics of intact animals cannot easily be explained by aversive inhibitory conditioning.

The appropriateness of describing leg lift learning as aversive inhibitory conditioning may depend on the degree to which flexion upon shock is an unconditioned, stereotyped response. If flexion occurs rapidly upon shock administration (which is common in headless animals) then inhibition of the leg extension component could produce the learned flexion response. However, if flexion is not an immediate stereotyped response (which appears more often to be the case for intact animals), then a more complex type of learning, involving exploration of the consequences of leg position and a newly formed shock reaction would be required to achieve the learned response.

However, it should be remembered that the validity of classifying leg position learning in the ventral nerve cord as aversive inhibitory conditioning has not yet been demonstrated (see Alloway 1973; Davis, 1975) and whether there are aspects of two different types of learning operating in headless and intact animals cannot be determined on the basis of present evidence. However, it is a possibility that should be considered when headless or isolated ganglion preparations are used as model systems to investigate mechanisms of learning and memory.

Experiments comparing the behavior of intact, headless,and isolated ganglion preparations allow for the possibility of dissecting out various components which may contribute to the final learned behavior of an animal. It is, of course, possible that the learning seen in the ventral nerve cord preparation is fundamentally different than that occurring in the intact system. It is also possible that the final learned behavior consists of many components, and lesioning is one way of dissecting out the role of various parts of the nervous system in determining behavior. The possibility that "cerebral" and "ganglionic" types of learning may be used by the animal for different situations should also be considered. For example, learning postural or locomotor adjustments may be mediated by ventral nerve cord ganglia, while learning to locate food or avoid predators may be mediated principally by the brain.

SUMMARY

The experiments reported in this dissertation were performed to investigate effects of CXM on learning and memory in the cockroach. The results confirmed that CXM impairs acquisition of leg lift training in headless cockroaches, and demonstrated that CXM has no impairing effect on acquisition in intact cockroaches trained to lift a leg or to turn left or right in a T-maze. Thus, the CXM impairment effect is limited to the headless preparation.

Experiments were performed to explore the nature of the CXM-induced impairment, i.e., whether it is an effect on the learning process which operates in headless cockroaches, or an effect on activity levels of the leg. CXM increased the leg activity (as measured by the number of dips) of the P animals (which would support an activity effect), but had no observable effect on the leg activity of the R animals (which would support a learning effect). No gross effects of CXM on the twitching threshold of the leg or on evoked electrical activity of peripheral nerves were detected (an observation which would be consistent with a learning effect). In view of the conflicting results, further proposed experiments are necessary to resolve the question of the nature of the CXM impairment.

The effects on retention of CXM injected before training were investigated. Injections of CXM which inhibited protein synthesis in the nervous system by over 90% did not produce memory deficits in

cockroaches tested at intervals up to 1 day after T-maze training. This is in marked contrast to the severe memory impairment observed in mice after injections of CXM which produce similar degrees of protein synthesis inhibition. Additional proposed experiments are necessary to determine what effects, if any, CXM and other protein synthesis inhibitors have on retention in cockroaches.

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