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THE EFFECTS OF PRE- AND POST-TRAINING ADMINISTRATION OF PUROMYCIN AND SCOPOLAMINE ON SHOCK AVOIDANCE LEARNING AND RETENTION IN THE COCKROACH, PERIPLANETA AMERICANA

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By

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ABSTRACT

THE EFFECTS OF PRE- AND POST-TRAINING ADMINISTRATION OF PUROMYCIN AND SCOPOLAMINE ON SHOCK AVOIDANCE LEARNING AND RETENTION IN THE COCKROACH, PERIPLANETA AMERICANA

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A one-session training procedure for cockroaches, in which animals are trained to turn right or left to avoid an electrical shock was utilized to investigate the effects of puromycin (PURO), a protein synthesis inhibitor, on learning and memory. The drug was injected three hours before training or three hours before testing in doses causing approximately 70 percent protein synthesis inhibition of the central nervous system. The number of correct turns, the number of trials to a criterion of five out of six correct responses, the time taken for an animal to proceed down the runway (runway time), and the time taken to proceed from the end of the runway until a choice was recorded (choice point time), were the behavioral parameters measured in this paradigm. In control animals the mean number of correct choices and the mean runway time both increased with succeeding trials during training. In addition, control animals showed excellent retention of these responses five hours later during testing. Puromycin, when administered before training, also had no effect on acquisition of these two responses. However, upon testing, these animals showed significant retention deficits of the correct turn and trials to criterion responses but not of the behavioral modification evidenced by increased runway time. Similarly, post-training injections of

PURO also caused some amnesia of the correct choice and trials to criterion responses. Thus, PURO may show specificity for the different types of behavioral plasticity that occur in any training situation and that may be mediated at different levels of the central nervous system.

Previous work utilizing the same paradigm and doses of cycloheximide (CXM) causing over 90 percent protein synthesis inhibition failed to produce retention deficits of either the correct choice or trials to criterion responses. Therefore, the absolute amount of protein synthesis inhibition cannot be the major causal factor in the PURO-induced amnesia.

In an effort to explain the amnesic actions of PURO in terms of its possible effect on cholinergic transmission, a series of experiments were performed in which animals were injected with scopolamine, a predominantly central muscarinic blocker, either one hour before training or one hour before testing in the same paradigm. Preliminary results indicated that the scopolamine-treated animals learned and retained the training as well as the control animals run with these experimental groups.

The PURO and scopolamine data together suggest that PURO is not interfering with central cholinergic synapses of the cockroach to produce its amnesic effect.

Dedicated to my Parents,

Anthony R. and Violanda A. Barraco,

who provided the means and
environment to pursue my life's goals,
with gratitude, admiration, and love.

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INTRODUCTION

If there is one quality that most aptly distinguishes the animate from the inanimate it is the former's capacity to modify behavior according to experiences encountered. This characteristic is referred to as behavioral plasticity (Davis, 1976a). Presently, four types of behavioral plasticity are known to be amenable to cellular analysis: habituation, choice, sensitization, and associative learning. This dissertation shall focus on pharmacological aspects of the most diverse, advanced, and intriguing form of behavioral plasticity; associative learning, and its companion phenomenon, memory.

The investigation will utilize a simple neurological systems approach to the problem with the experimental animal being the cockroach, Periplaneta americana. The justification of this experimental approach is readily apparent if one considers that one of the major obstacles encountered when studying the neurophysiology of learning and memory is the enormous complexity of the vertebrate nervous system. As an essential prerequisite for the investigation of the cellular and biochemical correlates of learning and memory, one ultimately needs to localize the physiological processes producing the behavioral change in a mass of tissue small enough for practical and meaningful analysis. This would be a formidable, if not presently insurmountable, task in a vertebrate nervous system with 10^{10} neurons or more. For this reason invertebrates, like the cockroach, offer an attractive alternative in which to investigate the cellular basis of learning and memory processes. The typical invertebrate nervous system possesses on the

order of 10^5 to 10^6 neurons, 80 percent of which are involved in analysis of sensory input. Therefore, the number of neurons responsible for the integration of motor output is relatively small; yet some feel it is among these few cells where one might expect changes associated with learning and memory to be located (Alloway, 1973). In addition, unlike vertebrates, invertebrates possess a "distributed intelligence," that is, their neurons are arranged in numerous discrete ganglia which are often homologous in structure and function. These so-called "mini-brains" may contain as few as nine neurons, however, a more typical number are several hundred to a few thousand (Eisenstein, 1972). Thus, the segmented invertebrate nervous system offers the prospect of localization of at least some forms of learning and memory, which suggests that it may be possible to approach a cellular understanding of these phenomena. Finally, invertebrates possess nerve cells that are often much larger than vertebrate neurons and brightly pigmented. This allows easy visualization and penetration with stimulating and recording electrodes. Also, because single neurons occupy the same position and have the same synaptic connections in all members of a given species, this permits repeated access to the same cell (Davis, 1976a).

There are two major experimental strategies that one may employ in defining any molecular involvement in processes such as learning and memory. One method would be to directly measure the suspected biochemical changes (e.g., protein synthesis, neurotransmission) during or following learning and memory. This could prove to be difficult if the suspected changes are small or somewhat undetectable by available analysis. The other approach would be to either attenuate or magnify

the biochemical processes by pharmacological agents (e.g., protein synthesis inhibitors, potentiaters or blockers of neurotransmitters) and see what effect such action has on the phenomenon being investigated. This experimental strategy is a less difficult course to follow. However, it leads to a more complicated interpretation of the data because it is often impossible to affect only the one physiological system, or portion of that system, that is to be studied. Nevertheless, the major impetus for this research was to acquire insight into molecular aspects of learning and memory processes. Thus, a thorough investigation utilizing the latter experimental strategy could serve many useful purposes, the greatest of which would be to lay a groundwork of pertinent questions that could only be answered by further biochemical studies.

LITERATURE REVIEW

Comparison of Invertebrate and Vertebrate Learning and Memory

Definitions and Examples of Learning and Memory Across Phyla

Associative learning may be defined as a behavioral modification caused by reinforced (rewarded or punished) experience; it includes the categories of classical (Pavlovian) conditioning and instrumental (operant) learning. The ability for longer-term associative learning is by no means unique to vertebrates. Invertebrates in which associative learning has been exhibited include platyhelminthes, annelids, and noncephalapod mollusks (for review see Corning, et al., 1973). Furthermore, Krasne (1973) points out that by the time one reaches the arthropods (e.g., cockroach) the capacity for associative learning not only is unmistakedly demonstratable but in many cases is well known to play a clear and useful purpose in the animals' day-to-day lives. "Members of this phylum utilize their learning abilities to aid activities such as foraging, homing, and the establishment of stable dominance in much the same manner as mammals do. Even behavior which in mammals is sometimes thought to be mediated by internal, self-produced cues or to be signs of 'higher mental processes' have been reported" (Krasne, 1976). Thus, the presentation of associative learning capabilities of invertebrates that follows is offered to demonstrate that invertebrates do possess the neurological machinery and the potential for disclosing mechanisms of associative learning and memory.

In classical conditioning, two sensory stimuli (the unconditioned stimulus, or US, and the conditioned stimulus, or CS) are presented together with the CS preceding the US. Usually, after few presentations the response formerly caused only by the US (i.e., the unconditioned response, or UR) can also be elicited by the CS alone (i.e., the so-called conditioned response, or CR). Classical conditioning has been demonstrated in most of the invertebrate phyla including platyhelminthes, annelids, arthropods, and mollusks. (For reviews see Davis, 1976b; Corning, et al., 1973; and Eisenstein, 1967). For example, in the gastropod, Pleurobranchea, Mpitsos and Davis (1973) paired touch with food stimuli until the formerly aversive touch stimuli became capable of causing the feeding response. Such behavior lasted up to two weeks without reinforcement, before extinction was complete.

Classical conditioning experiments also have been reported, but less frequently, in insects. In one study by Nelson (1971) Drosophila were presented with a compound CS, consisting of the animal's feet in water followed by contact of the feet with a salt solution. This CS was combined with a US consisting of sugar solution applied to mouth parts, which normally elicits extension of the feeding probiscus (the UR). Eventually the flies responded with the probiscus-extension response to the compound CS alone.

Instrumental learning resembles classical conditioning in that the behavioral modification is caused by a temporal association of two events. However, it differs in that one of the two events (the reinforcement) is not inevitable, as in classical conditioning, but instead

is contingent upon the animal's voluntary or spontaneous behavior. In instrumental learning the reinforcement may be positive (rewarding) or it may be negative (punishing).

Avoidance conditioning as an example of instrumental learning also has been studied in the Pleurobranchea by Mpitsos and Davis (1973). The authors shocked classically conditioned animals whenever they exhibited the classically conditioned feeding response to touch described above. Animals quickly learned (one day, 20 trials) that touch was associated with the aversive shock and ceased feeding to the touch stimulus alone. Also, Mpitsos and Collins (1975) found that Pleurobranchea presented with squid homogenate and simultaneous electrical shock soon learned to suppress the feeding response to the squid homogenate. This learned response persisted for more than eight days.

Quinn, et al., (1974) demonstrated that the insect, Drosophila, also is capable of associative learning. Populations of the flies were presented with two different odors. One was accompanied by an aversive electrical shock while the other was presented alone. The flies learned to selectively avoid the odor associated with the aversive stimulation. The task was acquired in one trial and retained up to one day. Control procedures eliminated the possibilities of habituation, sensitization, odor preference, and experimental bias.

One of the best known and most intensively studied examples of associative learning in invertebrates is the instrumental avoidance learning of leg position in the cockroach. This paradigm was first described by Horridge (1962) and later investigated by others without significant alteration in experimental design. In the basic paradigm

the animal is suspended above an electrical saline solution with a wire electrode attached to one of its legs. When the leg extends, the electrode makes contact with the saline solution, completing an electrical circuit and results in the animal receiving an electrical shock which causes leg flexion. Yoked controls are shocked by the same stimulus regardless of their leg position. Under such conditons the experimental animals learn to flex their legs against the pull of gravity to avoid the shock. In contrast, control animals do not exhibit this learning. Most of the leg lift experiments have been performed on headless animals, demonstrating that learning can occur in a ventral nerve cord, as well as being retained for up to 72 hours (Eisenstein, 1972). In addition, Eisenstein and Cohen (1965) demonstrated that the leg lift learning can be mediated by an isolated thoracic ganglion (approximately 1,000 neurons) thus, opening the possibility of further localization of learning and memory processes.

In related cockroach avoidance learning experiments using intact animals, Szymanski (1912) and Turner (1912) utilized a dark avoidance learning paradigm. The apparatus consisted of a closed box with a darkened and lighted side. The normal reaction of the cockroach when positioned on the lighted side was to immediately enter the darkened side, since cockroaches prefer dark to light. However, by electrifying the floor of the darkened side the authors were able to rapidly teach the animal to avoid its natural choice and stay positioned on the lighted side.

Maze training has also been used to train cockroaches. Turner (1913), Eldering (see Guthrie and Tindall, 1968), and Longo (1964) all used simple mazes in which the animals were trained to avoid one side

of the maze to avoid either an electrical shock or a bright light as a negative enforcer. It appears from these and other studies that negative reinforcement has proven to be a very reliable method for cockroach training.

Longer- and Shorter-Term Memory Stages

Kandel (1976) has defined memory, in the broadest sense, as the ability of an animal to retain or store a behavioral modification. Thus, memory can be assumed to have occurred in any of the above examples where animals were trained either in classical or instrumental procedures and later showed retention of the training experience. Although many researchers support the notion that all memories are produced by the same mechanism, the evidence is not compelling (Kandel, 1976). Different types of learning may give rise to different storage and/or retrieval mechanisms. The molecular relationship(s) between learning and memory mechanisms remains an important and largely unresolved issue.

Nevertheless, there are further similarities between invertebrate and vertebrate memory, as well as learning, processes that make invertebrate systems attractive alternatives in which to study these phenomena. One is evidence suggesting a two-stage memory process in invertebrates, resembling that of vertebrates. Kamin (1957) observed that retention of dark avoidance learning in rats decreased to a minimum at one hour after training and subsequently increased over a period of the next 19 days. This "Kamin effect" has been interpreted as being indicative of a transition period between two stages of memory, shorter- and longer-term. At the lowest point of retention it is postulated that

shorter-term memory has decayed and longer-term memory has not yet been fully established. A similar pattern of retention, with a minimum retention at one hour, has been observed by Eisenstein (1970) who trained headless cockroaches to avoid an electrical shock in the leg-lift paradigm. In addition, a "Kamin effect" has been observed in grain beetles trained in a maze (Alloway, 1969).

Retrograde amnesia studies also present evidence for the existence of a dual stage memory process in invertebrates. In these experiments, which are common in vertebrates, retrograde amnesia is observable if any one of many physical or chemical agents (e.g., cold, CO_2, electroconvulsive shock, anoxia) is administered immediately after training. Amnesia is not observable if the agent is administered some period of time (which may vary from seconds to hours depending on the agent used) after training. Results again have been interpreted as suggesting "consolidation"of the memory trace from a shorter-term to a longer-term stage such that the disruption of memory by certain agents may occur during the unstable shorter-term stage, but not during the permanent longer-term stage. Such phenomena has been observed in cockroaches. Minami and Dallenbach (1946) reported that treadmill running interfered with retention in the cockroach only if the running was administered immediately after training. If the treadmill running was administered one hour after training there was no effect upon retention. Carbon dioxide (an anesthetic which causes convulsions before quiescence) also causes retrograde amnesia in cockroaches. Freckleton and Wahlsten (1968) observed that if CO_2 was administered immediately after daily

learning trials passive avoidance was prevented. Similarly, Lovell and Eisenstein (1973) administered CO_2 immediately after or one hour after dark avoidance training and measured retention two hours after training. Immediate CO_2 administration caused amnesia; however, when CO_2 was given one hour after training some retention was observed. Thus, invertebrates, and in particular cockroaches, exhibit memory stages of similar time courses as that observed in vertebrates. In addition, there is similar susceptibility to disruption. This would imply that invertebrates present a useful model in which to investigate these memory stages.

Biochemical Correlates of Learning and Memory

The information that constitutes the conditioned and unconditioned stimuli enters the nervous system in the form of electrical signals in sensory neurons. Further, the expression of memory must eventually be demonstrated in the form of motor output through the activation of efferent pathways. Thus, given that electrophysiological correlates of learning and memory exist, it may be asked how such correlates are related to the actual learning and memory processes. Some believe that these electrical signals entering the central nervous system may trigger biochemical synthesis in central neurons, and that these biochemical changes may be imagined to underlie learning and memory processes in three distinct ways (Davis, 1976b). First, learned information that enters electrically is also retained as a memory trace in the form of electrical messages (action potentials) that continually reverberate in interneuronal "storage" circuits. Second, biochemical changes may

mediate structural and functional changes (i.e., more vesicles) of neurons that participate in the learned behavior, rendering such neurons more effective in subsequent trials. Third, information entering the brain in the form of electrical messages may in some way trigger the synthesis of macromolecules, the structure of which could uniquely code for behavior.

Davis (1976b) presents three lines of evidence that lend support to any biochemical hypothesis. First, and the least controversial, de novo biochemical synthesis can be shown to accompany learning. Second, chemical agents that block biochemical synthesis of proteins or neurotransmitters, prevent neurotransmitter release, or increase the level of specific neurotransmission have been shown to attenuate or facilitate learning and memory processes, respectively. Third, and the most controversial, there are reports that when macromolecules from trained animals are injected into untrained animals the recipient animals behave as if trained. That is, they learn the specific task for which the donor animals were trained faster than controls, or they exhibit the learned behavior without prior training.

Since the present study will assess the effects of manipulating the level of protein synthesis and neurotransmission on learning and memory processes, a review of the literature associated with "the second line of evidence" for both vertebrates and invertebrates will be presented.

Use of Antibiotics in the Investigation of Memory

Antibiotics have been used as amnesic agents in different species using a variety of learning paradigms (for review see Barraco and

Stettner, 1976). The initial interest in these drugs stemmed from their effects on protein synthesis; they were viewed as an avenue for exploring various hypotheses concerning the role of protein synthesis in memory formation.

There have been a number of reports on the effects that antibiotics such as puromycin (PURO), cycloheximide (CXM), and actinomycin-D, have on memory function. The research has been done predominantly on vertebrates, with particular emphasis on rodents and goldfish. It is generally accepted that these agents have disruptive effects on memory (i.e., failure to perform a previously learned task) if administered at the appropriate sites (intracranial or intraperitoneal injection), and at the appropriate times (preceding or following training). What is not well established is the interpretation of these results. All of the above drugs inhibit protein synthesis (through different mechanisms) and all have been shown to disrupt memory as shown by production of amnesia (Barraco and Stettner, 1976). Therefore, it has been concluded that memory formation (consolidation) and/or memory recall (retrieval) must involve peptide or protein formation. But this conclusion becomes less clear when it is noted that no drug has a unique effect on, or specificity for, a single physiological process such as learning or memory.

However, the deduction that memory function and peptide or protein formation are closely related may not seem unreasonable since memory can be a relatively permanent phenomenon. For such a permanent change to occur one would have to assume that at least minimal structural changes would take place to account for this stability. This then might be accomplished by at least some protein formation. But to say

that memory is simply a product of peptide or protein formation, as certain authors apparently advocate, appears unwarranted at this time.

Two main processes are involved in the production of protein from the genetic code: (a) transcription, in which mRNA is synthesized from a DNA template, and (b) translation, in which a polypeptide is produced based on the mRNA sequence. The general process of translating the RNA sequence into protein may be divided into four major reactions (Barraco and Stettner, 1976): (1) formation of the amino acid-tRNA complex (aminoacyl-tRNA); (2) interaction between ribosome, mRNA, and the aminoacyl-tRNA to form a ternary structure; (3) synthesis of peptide bonds involving the enzyme(s) peptide synthetase; and (4) translocation of the ribosome along the mRNA strand. An antibiotic that inhibits any one of these processes will inhibit protein synthesis. Antibiotics that primarily inhibit translation are those which have been found to be the most useful experimentally. Those agents that inhibit peptide formation by a primary inhibition of DNA or RNA synthesis are not as useful due to their extreme toxicity.

Three major classes of antibiotics have been utilized in these investigations: the antinomycins, the glutarimides, and the amino-nucleosides. Each of these classes of pharmacological agents exerts its effects on protein synthesis through different mechanisms. Similarly, each drug produces other biochemical effects in addition to inhibiting protein synthesis.

The glutarimides (e.g., CXM) are presumed to interfere with movement of ribosomes along the mRNA strand, thus preventing translation. These

drugs appear to interfere with the interaction between the enzyme peptide synthetase and the ribosome. The precise mechanisms are not known (Beard, et al., 1969; Sisler and Siegal, 1967).

The aminonucleosides (e.g., PURO) inhibit protein synthesis by interfering with mechanisms involved in translation. It is suggested that agents in this group act as analogs of aminoacyl-tRNA and therefore accept the growing peptide chain. Once incorporated into the growing chain the peptide is released from the ribosome as a peptidylpuromycin fragment (Nathans, 1967).

Actinomycin-D prevents DNA transcription. It is no longer an accepted agent with which to disrupt memory and therefore literature pertaining to its use will not be reviewed here.

Antibiotic Experiments on Vertebrates

The result that antibiotics cause amnesia of learned tasks in vertebrate systems has been known for the past 15-20 years. These studies differ in many ways--antibiotic used, dose level, injection location, animal species, type of learning task, and retention measure, extent of original training, temporal relationship between training and antibiotic used, time interval between injection and retention test, and the interval between training and the retention test (Barraco and Stettner 1976). These are by no means all of the critical variables that may be important in the production of experimental amnesia. Thus, an attempt will be made to concentrate on the major findings that receive support throughout the literature as well as those that contribute to the few common theories that exist in this area of research.

Vertebrate Puromycin (PURO) Studies

Successful experiments in which puromycin (PURO) was used to produce amnesia in experimental animals began with the studies of Flexner, et al., (1963). These investigators found that intracerebral (temporal lobe) injections of PURO in mice producing 80-90 percent inhibition of protein synthesis 1-3 days following training in a shock avoidance Y-maze led to a loss of memory when animals were tested a few days after the injections. However, the animals receiving PURO treatment were able to relearn the maze and were capable of reversal learning. This new learning was retained indefinitely. No amnesia effect was observed in the mice receiving this type of injection five days or more after training. Bilateral injections of PURO at sites that produced inhibition of protein synthesis over the entire cerebrum also produced amnesia (but again had no effect upon learning) even when the injections occurred several weeks following training. In either situation the amnesia was not immediate but required 12-20 hours to develop. Once established, the amnesia persisted for three months (Flexner, et al., 1967; Flexner, et al., 1964; Roberts and Flexner, 1969).

Barondes and Cohen (1966) carried out a set of experiments in mice using a similar shock avoidance paradigm as Flexner and his co-workers. These authors found that injections of PURO 1-5 hours before training had no effect on learning or acquisition to a criterion of nine correct responses in ten trials. However, upon testing 2-3 hours after training the animals injected with PURO showed a progressive decrease in retention.

Mayor (1969) found that intracerebral injections of PURO in quail immediately following red-green color discrimination produced retention

deficits during testing for the same discriminations three days later. Puromycinaminonucleoside, a drug which shares many of the effects of PURO on carbohydrate metabolism, etc., but has no effect on protein synthesis, and saline had no effect. Mayor (1973) demonstrated similar effects of PURO on the retention of pattern discrimination in quail.

Mark and Watts (1971) investigated the effects of PURO on retention of a one-trail passive avoidance task on one-day-old chicks. The authors employed a banana plug coated with methylanthranylate as an aversive pecking stimulus so that an absence of pecking after initial exposure to the plug signified retention. Injections were made either three hours before training (so as to allow training to occur during the maximum inhibition of protein synthesis) or five minutes before the beginning of training trials. The dosage was either 90 or 180 μg of PURO. Retention testing was conducted at intervals of 10, 30, 60, or 90 minutes as well as 24 hours after the initial trial. Ten seconds of pecking at the bad tasting plug during the single trial was sufficient to produce inhibition of pecking for almost 100 percent of the untreated control chicks over the entire 24 hour interval. In all cases, the PURO-injected animals showed a progressive decay of memory over the 90 minutes of testing following training, whereas the saline-injected control animals exhibited stable retention over the same period. There was also evidence of memory loss at 24 hours in all PURO-injected groups.

Puromycin also interferes with memory processes in fish, but there are important differences in its actions in these animals from those discussed above for mammals and birds. In experiments conducted on shock avoidance in goldfish, PURO, when injected intracranially immediately

following training produced a marked decrease in avoidance responding three days later. A similar amount of PURO given 1-2 hours after training produced no amnesia three days later (Agranoff, 1968, 1970; Agranoff, et al., 1965, 1966). This need for immediate injections of PURO to produce retention deficits in fish has been substantiated by others (Braud and Broussard, 1973; Neale, et al., 1973) and is in sharp contrast to the frequent findings of amnesia in mice that can be produced with delayed injections of 24 hours or more after training (Flexner, et al., 1963, 1964, 1967; Roberts and Flexner, 1969).

Vertebrate Cycloheximide (CXM) Studies

Flexner and Flexner (1966) Flexner, et al., (1967) administered acetoxycycloheximide (AXM) or cycloheximide (CXM) intracerebrally in mice trained in the same paradigm as for their PURO studies. Unlike the results with PURO, injections of these two glutarimides 1-3 days after training had no effect on memory. However, when animals were given intracerebral injections 2-4 hours before training, the animals showed (1) an impaired capacity for learning during training, and in addition, (2) memory of the training which was evident at three hours gradually decayed only to return 3-4 days later. When mice were injected with AXM immediately after training, memory of the training persisted for a period that extended to 14 hours followed by a period of impaired memory. Again the memory returned to normal after 3-4 days (Flexner, et al., 1966; Roberts and Flexner, 1969).

Barondes and Cohen (1967a) found that mice injected in the temporal lobes with AXM ten minutes to five hours before training to avoid shock showed no memory impairment three hours after training but were amnesic

six hours after training and thereafter. Identical injections given 18 hours before, immediately after training, or four days after training had no effect on retention of the learned task. The authors also demonstrated two variables that are essential to the glutarimide induced amnesia: (1) the level of training and (2) the degree of protein synthesis inhibition. The authors found that if training was conducted to a criterion of nine correct responses out of ten instead of three out of four that the drug produced either a transient amnesia that lasted only 5-14 hours, or that the drug had no effect at all upon retention of the learned task. Similarly, the degree of protein synthesis inhibition also determined any observable behavioral effects--retention deficits were produced only when the degree of protein synthesis inhibition was over 90 percent. Amnesia was not produced with doses of AXM that caused 80 percent protein synthesis inhibition or less.

Barondes and Cohen (1968a) later employed subcutaneous injections of AXM using 12 times the intracerebral dose in mice trained for light-dark discrimination. This injection regime caused a very rapid and large protein synthesis inhibition (90 percent after ten minutes) which remained for eight hours. Mice were trained to a criteria of five correct responses out of six. When injections were made five hours to five minutes before training the animals showed a marked amnesia when tested. Injections made immediately before, immediately after, or five minutes after training caused a lessened degree of amnesia, while no impairment of memory was observed if the injections occurred 30 or more minutes after training. In all situations where an impairment of memory was evidenced it did not appear during retention testing three hours after training but was apparent seven days after training.

Daniels (1971) injected AXM into the hippocampi of rats in doses which produced 95 percent protein synthesis inhibition during training in a shock motivated, brightness discrimination task. The injections were given five hours before training. Learning was unaffected. However, memory, which was not affected three hours after training, was severely impaired at six hours, 24 hours, and seven days after training. When injections were given five hours before testing or immediately after learning trials retention was normal.

Mayor (1969) found no evidence of amnesia when CXM or AXM was injected immediately after training quail in the same discrimination learning paradigm that the author used for injections of PURO in which retention deficits were observed.

Comparison of Vertebrate Puromycin (PURO) and Cycloheximide (CXM) Studies

Puromycin-induced memory loss has been found in rodents, birds, and fish. The majority of these studies have employed shock avoidance paradigms. However, there also have been studies utilizing taste aversion in a passive avoidance task, positive food reinforcement, and discrimination tests in which similar results have been obtained. Amnesia is consistently produced when these injections take place 24 hours or more after learning, as well as, when they are given up to five hours before training or immediately after. While having a potent affect on memory PURO does not appear to affect acquisition. In some studies it does appear that the degree of learning is a variable in the quality of the amnesia produced but it does not appear to be as critical as with the CXM injections (Barraco and Stettner, 1976).

In marked contrast to the PURO findings, injections of CXM or AXM typically fail to produce amnesia when injected any time after learning. However, the glutarimides do consistently produce retention deficits when administered immediately before and up to five hours before training. As with PURO the majority of these experiments involve rodents and shock avoidance paradigms. But amnesia also has been seen with studies utilizing passive avoidance, food and water reinforcement, and discrimination training procedures. The degree of training has been shown to be a very critical variable in producing amnesia (Barondes and Cohen, 1967a; Flood, et al., 1972). It also has been found that the amnesia effects produced by the glutarimides develop over time. That is, animals may continue to respond in a well-trained manner as much as three hours after initial training after which the animals typically begin to progressively demonstrate a decline in retention with a peak loss often occurring six hours after training. As with PURO, CXM and AXM do not appear to affect learning processes.

The original and still major guiding premise of the antibiotic work has been that these agents interfere with memory by inhibition of protein synthesis that is in some way necessary for the development and maintenance of longer-term memory storage or fixation (Agranoff, et al., 1978). However, it is known that these drugs have other physiological effects which could produce behavioral abberations. In addition, the quality or type of amnesia produced by PURO and CXM differs as to length, time course of development, and susceptability of alteration by other drugs and behavioral procedures. Thus, it is necessary to consider other factors before drawing any conclusions as to the mode of action of these agents on memory processes.

Side Effects of Antibiotic Treatments

Antibiotics, in doses that are required to produce retention deficits do produce side effects. For example, Flexner and Flexner (1967) noted that PURO has toxic side effects such as lowered activity, drowsiness, loss of alertness, failure to eat or drink, and weight loss. These effects appear to be dose related. In addition to these general toxic effects, Cohen and Barondes (1967) and Cohen, et al., (1966) found that PURO produced diminished and irregular electrical activity in the hippocampus of mice. They also found a possible synergistic effect of PURO and convulsion-inducing drugs. Further, Flexner and Flexner (1968) found evidence that pepidyl-puromycin fragments, which are thought to have some deleterious effects themselves, survive for at least several weeks in the systems of mice.

The glutarimides also have toxic effects. Flexner and Flexner (1966) report that diarrhea is a common symptom in mice, and that these animals often fail to eat or clean themselves for two or three days after injection. Barondes and Cohen (1967a) found that CXM produced activity changes in mice. It also was noted by Randt, et al. (1973) that CXM produces alteration in the electrical activity of the brain in very specific locations. In addition, CXM produces alteration of such established behaviors as nest building (Schneider and Chenoweth, 1971). This latter effect was seen to last beyond the time that was necessary for protein synthesis to return to normal. Furthermore, the glutarimides as well as PURO disrupt polysomes. However, the population that is affected by each is different (MacInnes and Luttges, 1972, 1973). Thus, it can be seen that injection of either class of

antibiotic can be associated with side effects in addition to their effects on protein synthesis. Therefore, one must exercise caution when asserting causality between the behavior changes observed and biochemical events produced by the drugs.

However, it should be stressed that within the dose range utilized for either class of drugs animals typically do recover and behave in a normal manner within several days after injection. In addition, experimental designs in most situations are able to control for any "sickness" variable. For example, one approach to the sickness problem that has been employed is to demonstrate that the treated animals are in fact capable of carrying out the required task at normal levels at the time of testing. This is accomplished by either showing their ability to acquire new learning or re-learning at the time of testing or demonstrate that the animals can perform adequately on some previously learned task that is not susceptible to treatment of the antibiotic injected, either because it was a very well-learned task or because it was acquired in a time frame that made it invulnerable to the treatment.

Permanent of Antibiotic-Induced Retention Deficits

There are reports which question the permanence of antibiotic-induced amnesia or demonstrate the ability to antagonize a drug's potential amnesic effect. These considerations are crucial because the most pertinent question raised by the retention deficit produced by these antibiotics is whether the amnesia produced represents interference with the fixation and storage mechanism or whether the retrieval process is impaired, with the fixation and storage mechanism being left intact. It has been assumed that interference of the fixation mechanism(s)

either by prevention of consolidation of memory from shorter-term to longer-term storage or disruption of already existing consolidated longer-term memory should produce a permanent amnesia. Conversely, recovery of previously lost memories implies that the storage mechanism must be intact and any amnesia produced must have been due to interference with the retrieval process. Thus, studies observing recovery from antibiotic-induced amnesia is important because they cause one to focus on pertubation of the retrieval process in explaining the retention deficits observed. In addition, the conditions which lead to memory recovery help elucidate mechanism(s) that may underlie the retrieval process.

There have been no reports of spontaneous recovery from PURO-induced amnesia. As stated by Barraco and Stettner (1976) the fact that animals are often tested 3-4 days after treatment and that one study (Roberts, et al., 1970) found PURO-induced amnesia was still present 18 days after treatment generates some degree of confidence to this statement.

The reporting of spontaneous recovery from CXM-induced amnesia is more controversial. Many authors report no evidence of spontaneous recovery after injection even when testing 1-3 weeks after treatment (Barondes and Cohen, 1967b, 1968b; Flood, et al., 1972). However, there are several investigators that do report spontaneous recovery (Flexner, et al., 1966; Serota, et al., 1972), and at least one of the authors that reported recovery (Squire and Barondes, 1972) also reported no recovery (Barondes and Cohen, 1967b, 1968b). All reports of spontaneous recovery of memory with CXM occurred when the drug was administered before training and memory deficits were found to be present 24 hours

after training but not 2-7 days later. All studies involved rodents and shock-motivated paradigms (i.e., two choice discrimination escape or one-trial passive avoidance).

In the studies that conflict as to finding spontaneous recovery of glutarimide amnesia it has not been possible to define a procedural difference between studies (Barraco and Stettner, 1976). However, regardless of the final determination as to the conditions that are responsible for the occurrence of non-occurrence of spontaneous recovery, these reports are significant because they suggest that the massive protein synthesis inhibition that is present during training in which CXM or AXM have been injected shortly before does not necessarily permanently block the memory consolidation process, while PURO will.

There also are situations where lost memories can be recovered by a variety of physiological and behavioral treatments. Puromycin-induced retention deficits are no exception. For example, Flexner and Flexner (1967, 1968) report that bitemporal injections of isotonic saline four hours to 60 days after PURO administration restores the memory normally eradicated by intracerebral injections of PURO 1-3 days after training. These results were confirmed by Rosenbaum, et al., (1968) who were able to reverse the retention deficits produced by injections of PURO one or more days after training. Conversely, these same authors were unable to reverse the amnesic affects of injections of AXM that were given five hours before training. Saline injections are either ineffective or slightly effective in reversing PURO-induced amnesia if the drug is given before or immediately after training (Flexner and Flexner, 1968).

Drugs that stimulate the adrenergic systems also act to produce recovery from some antibiotic-induced retention deficits. Roberts, et al., (1970) injected PURO into mice one day after training. This was followed eight days later by injections of either imipramine, tranylcypromine, or D-amphetamine in maximum tolerable doses. Ten days after these injections animals were tested for retention. Memory was restored in most of the animals injected with the stimulant drugs.

Barraco and Frank (in preparation) also found PURO-amnesia antagonism with injections of amphetamine. These authors discovered that simultaneous injections of PURO and D-amphetamine one day after training prevented amnesia in mice tested four and seven days after injection. However, if simultaneous administration of the two drugs was given immediately after training amnesia was apparent upon testing.

Serota, et al., (1972) detailed a similar antagonism of glutarimide-induced amnesia. In this study metaraminel and D-amphetamine both were shown to prevent AXM-induced amnesia. Also Barondes and Cohen (1968b) found that when injections of D-amphetamine in CXM-treated mice were given three hours after training (before amnesia) retention was normal at six hours and seven days after training, when retention deficits would ordinarily occur with CXM injections alone.

Glutarimides injected with PURO also has been shown to either prevent or diminish the amnesic effect of PURO. Flexner and Flexner (1966) and Barondes and Cohen (1967b) demonstrated that mice given both PURO and CXM or AXM drugs one or more days after training prevented or decreased any potential amnesic effect of PURO administered alone.

Similarly, in a series of experiments drawing striking parallels to their amphetamine and PURO work, Barraco and Frank observed that

simultaneous injections of PURO and CXM in mice prevented amnesia when given one day after training but did not prevent retention deficits when injected immediately after training.

Glutarimide-induced amnesia has been shown not only to recover spontaneously, and by administration of stimulants, but also by behavioral treatments. For example, when animals are given repeated exposure to the training apparatus or a "reminder shock," those animals that normally would display retention deficits appear to recover from the amnesia (Quartermain, et al., 1972).

To summarize, considerable evidence has been accumulated that demonstrates that some amnesia produced by PURO or by the glutarimides can be blocked or restored. The implication then must be that administration of these drugs within certain time frames neither totally prevents consolidation nor completely destroys longer-term memory. If true, a logical conclusion from the aforementioned situations where the actions of antibiotics are able to be antagonized might be that the drugs are producing retention deficits by blocking or impairing retrieval processes providing access to memories for specific aspects of a training experience. In addition, it should again be noted that many of the studies report no recovery from amnesia induced by administration of either PURO or the glutarimides, the most noteworthy being when PURO is injected before training (Barraco and Stettner, 1976). This has caused some authors to conclude that the administration of PURO before training, such that protein synthesis inhibition is maximal during acquisition, is interfering with fixation of memory; while effects of PURO long after training (one or more days) is perturbing the mechanism(s) involved in the retrieval of the memory trace. Injections of PURO immediately after training

may be interfering with both the consolidation and retrieval processes, and as such may be difficult, if not impossible, to separate behaviorally.

Summary of Antibiotic-Induced Retention Deficits

The effects of the two major classes of antibiotics on vertebrate learning and memory processes that have been discussed were summarized into three separate classes of behavioral effects by Barraco and Stettner (1976). They are: (1) PURO and CXM, when injected five hours to 30 minutes before acquisition training, produce a disruptive effect on memory which is apparent when the animals are retested. The CXM-induced amnesia is not apparent until six hours after training, while the PURO-induced retention deficits is present three hours after training. The CXM-induced amnesia in this time frame is either transient or can be attenuated with behavioral and/or pharmacological manipulation. This is not true for the PURO-induced amnesia which appears to be permanent and is not reversible by saline injections; (2) PURO when injected immediately before or immediately after training produces amnesia upon retesting. This amnesia appears permanent and is not reversed with saline injections or injection of stimulants. On the other hand, when glutarimides are injected either immediately before (less than five minutes) or immediately after (greater than five minutes) training, there is a lessened amnesic effect despite the fact that inhibition of protein synthesis reaches over 90 percent in less than ten minutes after injection. Furthermore, the slight amnesia produced in this time situation is evident only after brief training. Conversely, although PURO-induced inhibition of protein synthesis takes at least one hour to reach 90 percent, marked amnesia is apparent when injections occur

ten minutes after training; (3) PURO when injected at a later time after training (one day to several weeks), presumably after consolidation has occurred, prevents the retrieval or recall of the memory. This subsequent disruption of memory can be reversed with saline injections, certain stimulants of the adrenergic system, or simultaneous administration of CXM.

Neurotransmitter Modulation and Vertebrate Learning and Memory

Drugs that affect neurotransmitter systems are other pharmacological tools that researchers have employed in attempts at reaching a molecular understanding of learning and memory processes (Zornetzer, 1978; Alpern and Jackson, 1978). The major experimental strategy has been to increase or decrease the amount of a particular neurotransmitter before and/or after training and see what effect, if any, such treatment has on learning and memory processes. While simple enough in principle, such work leads to the inevitable ambiguities noted in the antibiotic work. That is, no single manipulator of neurotransmission has been shown to only affect neurons associated with learning and memory processes. As such, these types of drugs also may affect the processes of sensory registration, arousal mechanisms and/or motor output, that may or may not have important nonassociative effects on behavior that are unrelated to actual learning or memory. The extent to which the effects of drugs that manipulate neurotransmission are related to these nonassociative effects as opposed to actual learning, memory storage, or retrieval processes is a largely unanswered question. Thus, the reader should bear this in mind when interpreting the results of the studies to be detailed.

Vertebrate Cholinergic Studies

Acetylcholine has been the most widely studied central nervous system neurotransmitter in relation to learning and memory processes. The cholinergic synapse is most effectively modified through the use of various pharmacological agents that accentuate or attenuate transmission rather than manipulating specific brain regions. In general, it has been found that drugs that increase cholinergic activity enhance learning and memory processes while agents that block cholinergic transmission disrupt learning and memory mechanisms (Zornetzer, 1978).

A frequently employed method to increase cholinergic activity is to administer cholinesterase inhibitors such as physostigmine and diisopropylfluorophosphate (DFP). In one of the few reports of these drugs administered before training, Whitehouse (1966) found that within a small dose range physostigmine injected into both rats and cats prior to maze running and discrimination tasks facilitated acquisition of the training. Alpern and Marriott (1973) found similar results. The latter authors trained mice in a T-maze to reverse their position tendency (left to right) after a cue (shock or light, or both) presentation. The animals were then provided on each successive day following training with a sign-trial during which the cue controlling their behavior (go left or go right on the next trial) was presented. Animals were injected with either one or two doses of physostigmine prior to daily testing and the test trial was presented at varying intervals after training to determine the length of time that this information could be retained. Both doses of physostigmine improved performance at all testing intervals.

Stratton and Petrinovich (1963) and Greenough, et al., (1973) discovered that post-trial administration of physostigmine could also facilitate memory in rats trained in a Lashley III maze. In addition, these studies, taken together, make the point that the effect of physostigmine can interact with both genetic and environmental factors. That is, animals with a greater memory "efficiency" due to either genetic or environmental factors, required a lower dose of physostigmine to facilitate memory than animals without these inherited or environmental advantages.

In probably the most extensive study to date, Deutsch (1969, 1971) demonstrated that cholinergic synapses do participate in memory processes and that the effectiveness of specific transmission varies as a function of time. By injecting physostigmine or DFP into the hippocameal complex of rats, he was able to produce either facilitation or impairment of a learned response behavior with the same dose of the anticholinesterase agent by varying the time of injection after original training in a shock avoidance Y-maze paradigm. Performance was disrupted by injections given at 30 minutes and 3-14 days following training. It was unaffected at 24-48 hours, and improved at 28 days. Simplified, Deutsch concluded that as a result of learning the post synaptic endings at a specific set of synapses become more sensitive to neurotransmitter. This sensitivity increases with time after initial learning, then declines. He views memory as a pure strengthening of synaptic transmission in the cholinergic system and further that there must be a critical level of acetylcholine needed at memory synapses to permit them to function optimally. An insufficient amount of acetylcholine will prevent the "memory synapses"

from reaching the threshold necessary for firing, whereas an excess will cause a depolarization block.

The most extensively used anti-cholinergic drugs in studies of learning and memory processes have been the muscarinic-blocking agents atropine and scopolamine, with the latter apparently the more effective antagonist of central cholinergic synapses (Goodman and Gilman, 1975). Longo (1966), in an early review, summarized the known behavioral effects of the antimuscarinic drugs in a variety of different paradigms that utilized different species such as the rat, rabbit, dog, and monkey as follows: (1) little influence on discrete trial avoidance, (2) disruption of instrumental and operant reward conditioning and maze performance, (3) adverse effects on learning, and (4) lengthening of the extinction period of previously learned tasks.

Bohdanecky and Jarvik (1967) reported an impairment of one-trial passive avoidance retention in a step-through paradigm in mice given pre-training injections of scopolamine. Using a similar paradigm, Glick and Zimmerberg (1971, 1972) demonstrated that scopolamine could produce retention deficits in mice if injected prior to or within one hour after training. Furthermore, these authors found no retention loss when animals were injected within similar time spans with methylscopolamine, which blocks only peripheral muscarinic cholinergic transmission (Glick and Zimmerberg, 1972). Thus, the scopolamine-induced amnesia was concluded to solely involve a central rather than peripheral effect.

Both Deutsch (1969, 1971) and Alpern and Marriott (1973) used scopolamine to produce approximate mirror image results of their work with the anticholinesterases reported above. That is, scopolamine

generally inhibited memory formation when administered in the same time frames that injections of physostigime or DFP enhanced memory, thus further confirming the need for cholinergic transmission in memory fixation, either by acting directly on the memory trace or by affecting nonspecific processes that serve to modulate the memory trace.

Vertebrate Adrenergic Studies

Recent findings suggest that learning and memory processes also may be affected by manipulation of adrenergic synaptic transmission. As with cholinergic modification, the adrenergic synapse is most effectively altered by drug treatments that increase or block transmission rather than by manipulating specific regions of the central nervous system. Similarly, a particular pharmacological agent that affects adrenergic synapses can be found to have both positive and negative results on learning and memory processes. However, generally, drugs that increase catecholamine and dopamine levels have been found to facilitate learning and memory processes while inhibitors of these transmitters have been reported to have a negative effect.

The most effective method of increasing adrenergic transmission has been through the administration of amphetamine, which exerts its pharmacological action primarily through stimulation of catecholamine release. Krivanek and McGaugh (1969) found that both pre- and post-trial administration of amphetamine facilitated acquisition of a food-rewarded visual discrimination paradigm in mice "by affecting time-dependent memory storage processes." Furthermore, the results show that the drug's effects were not attributable to simply an "activity" effect. Costellano (1974) who trained mice to swim toward the light

or toward the dark in a Y-water maze, also noted that both pre- and post-trial administration of amphetamine had a facilitating effect on memory consolidation that was unrelated to performance or state dependent effects.

In an initial study Evangelista, et al., (1970) found that pre-trial injection of amphetamine in rats trained to avoid an electrical shock in a shuttle box increased performance of conditioned responses but did not improve retention of the paradigm; on the other hand post-trial administration of amphetamine did improve retention. In a follow-up study Evangelista and Izquierdo (1971) using amphetamine and atropine injections concluded that amphetamine has a dual effect on behavior: one enhancing pseudo-conditioning when injected before training; and another augmenting memory consolidation when administered after training. These actions, the authors concluded, were independent of one another and state dependent.

Reserpine, a drug that depletes the stores of catecholamines and serotonin (5-HT)in many organs including the brain and adrenal medulla, also has been used to determine the involvement of the adrenergic system in learning and memory processes. In a series of experiments Dismukes and Rake (1972), Rake (1973), and Allen, et al., (1974) used reserpine and concomitant administration of either serotonin or dopa postulate a pharmacological distinction for the types of memory formation that require the presence of either catecholamine or indoleamines (serotonin). These authors found that post-trial injections of reserpine resulted in retention deficits of different groups of mice given either passive avoidance or active avoidance training. If, however, serotonin was administered with the reserpine (thus decreasing catecholamines but

maintaining normal serotonin levels) retention deficits were observed in only the active avoidance group. When reserpine was given with dopa (thus decreasing serotonin but maintaining normal catecholamine levels) retention was normal for the active avoidance training group but not for the passive avoidance group. Thus, the authors concluded that catecholamines must be necessary for memory formation of active avoidance training and that indoleamines are necessary for memory formation of passive avoidance training.

When alpha-methyl-p-tyrosine (AMPT), a catecholamine synthesis inhibitor, is administered to rats there is a decrease in responding to rewarding electrical brain stimulation. When an agent is administered that selectively reduces serotonin levels behavioral responses are normal (Cooper, et al., 1971).

When 6-hydroxydopamine (6-OHDA), an agent that destroys adrenergic neurons, was administered with AMPT or reserpine, a deficit in a continuously reinforced bar pressing task in rats was observed. Injections of 6-OHDA alone did not cause any behavioral deficit (Cooper, et al., 1973).

In summary, adrenergic neurotransmission also appears to be an essential ingredient in memory. Whether it is acting on specific or nonspecific mechanisms related to consolidation and/or retrieval processes remains uncertain at this time.

Pharmacological Aspects of Invertebrate Learning and Memory

While there has been substantial research on pharmacological aspects of vertebrate learning and memory processes, there has been relatively little similar work on invertebrates. In addition, the small

number of studies investigating the effects of inhibitors of protein synthesis on invertebrate learning and memory contain important ambiguities and therefore are not particularly helpful in assessing any actual mechanisms of learning and memory.

Invertebrate Antibiotic Studies

Brown and Noble (1967, 1968) found that headless cockroaches treated with CXM in doses causing over 90 percent protein synthesis inhibition and trained in the leg-lift paradigm reached the same criteria as control animals but required a longer time to do it. These experiments support a theory, as put forth by the authors, that CXM impairs learning but not memory (i.e., no amnesia) in cockroaches. However, Eisenstein (1968) suggested that the learning impairment may be due to performance deficits rather than on specific aspects of the learning process per se. That is, CXM altered leg activity or sensitivity of the leg to shock. This hypothesis was supported in independent work by Glassman, et al. (1970) who trained headless animals for ten minutes, injected them either with CXM or saline, and resumed training five minutes after the injections. The performance of the CXM-injected animals was impaired during the retraining period in that these animals received significantly more shocks than the controls. Since both groups had previously reached criterion the effect of CXM was assumed to be on the performance of the leg-lift task rather than on acquisition processes.

Kerkut, et al. (1970) present data that also purports to show that CXM slows or impairs learning, but not retention, of cockroaches trained in the leg-lift task. These data are presented in the form of leg-position avoidance learning curves in which the number of shocks

to criterion and time to criterion are plotted. Davis (1976b) points out that neither of these parameters effectively distinguishes the effect of the drug on the rate of learning from its effect on initial activity levels. Davis believes that the essential data, the slope of the learning curve, is not presented. Thus, the authors conclusions as to the effect of CXM on learning and memory cannot be evaluated on the basis of the published data.

Emson, et al., (1971) report that injection of protein synthesis inhibitors such as cycloheximide and actinomyosin-D in the snail impairs avoidance learning. But the authors conclude that such results can be explained in terms of the toxic effects of the drug in producing desensitization of the animals to shock rather than blocking formation of specific "memory molecules." Other authors such as Davis (1976b) question whether the animals in the preceding experiments exhibited learning at all. This is again based on the fact that data are not presented that allows the reader to distinguish between the rate of learning and the initial activity levels.

Schwartz, et al., (1971) showed that protein synthesis inhibitors such as anisomycin, sparsomycin, and poctomycin that irreversibly inhibit protein synthesis up to 97 percent did not affect electrophysiological habituation in simple nervous systems, such as Aplysia, for up to 30 hours after injection.

More recently, Lovell (1975) has confirmed the results of Brown and Noble (1967, 1968) and Kerkut, et al., (1970) with regard to the effects of CXM on acquisition in the headless cockroach trained in the leg-life paradigm. Further, she tested the effects of CXM on learning and memory in the intact cockroach trained in the leg-lift task and

in the intact animal trained to turn right or left in a T-maze to avoid shock. In the T-maze experiments the drug had no effect on either acquisition or retention.

In summary, protein synthesis inhibitors have an unclear effect on acquisition of learning in the headless cockroach preparation and no effect on the intact preparation despite causing over 90 percent protein synthesis inhibition of the central nervous system. Thus invertebrate studies on the inhibition of macromolecular synthesis have contributed little to date in understanding the role, if any, that macromolecules play in learning and memory.

Invertebrate Neurotransmitter Studies

There also have been few studies aimed at assessing the role that neurotransmission plays in invertebrate learning and memory processes. Kerkut, et al., (1970), Emson, et al., (1971), and Kerkut, et al., (1973) present data that suggests that in the cockroach, drugs that increase the amount of neurotransmitter such as magnesium pemoline, D-amphetamine, physostigmine, and neostigmine all facilitate learning. However, these experiments suffer from the same criticisms that were made for Kerkut and his colleagues' work with CXM and as such must be viewed cautiously. A potentially much more interesting finding was that there is a rapid fall in the amount of cholinesterase in the cockroach with learning (Kerkut, et al., 1970, 1971, 1972; Oliver, et al., 1970). This suggests that as the animals learn there is increased activity of cholinergic synapses. These authors also found that as the levels of cholinesterase increase over a 72 hour period after training the animal begins to show retention deficits of the training experience.

However, Willner and Mellanby (1974) and Woodson, et al., (1972) both found no change in cholinesterase activity in the cockroach central nervous system utilizing the same paradigm and procedures as Kerkut, Oliver, and their co-workers.

Oliver (1973) reports facilitated learning in cockroaches injected with physostigmine and trained in the leg-lift paradigm. Evidence for this lies in the fact that control animals reached criterion in 30 minutes while animals injected with physostigmine reached criterion in 10 minutes. However, if one closely inspects the data it can be seen that the control animals learned in 26 minutes and the experimentals in 16 minutes. Further, it appears that the ten minute difference between the two groups can entirely be attributed to activity level differences apparent at the beginning of the experiment. The critical variable which would distinguish any difference in the rate of learning (i.e., the slope of the learning curves) between the two groups appears to be identical. The difference in activity levels can be accounted for by the fact that one group has been injected with a substance that increases cholinergic transmission in the central nervous system and which therefore would be expected to increase its activity (Davis, 1976b).

Thus, while it has been indicated that acetycholine functions in invertebrate learning (but apparently not memory) poor experimental design leaves the question as to the role of neurotransmitters in invertebrate learning and memory largely unresolved.

RESEARCH RATIONALE

The objective of this study is to determine what effect the antibiotic puromycin (PURO), a protein synthesis inhibitor, has on learning and retention in the cockroach, Periplaneta americana. These experiments will be performed within the framework of a search for the molecular basis of learning and memory. Puromycin, and other protein synthesis inhibiting drugs, such as cycloheximide (CXM), have been extensively used to produce amnesia (while not affecting acquisition) in vertebrates when administered both before and after training. The interpretation of these retention deficits has been that brain protein synthesis is required for some aspect of memory, either in the consolidation and/or retrieval processes. However, elucidation of molecular mechanisms of learning and memory has been thwarted by the enormous complexity of the vertebrate nervous system.

In contrast, very little research has been directed at exploring what effects these pharmacological agents have on invertebrate learning and memory processes. Such work would be warranted for at least two reasons:

1. the simpler nervous system that invertebrates possess more realistically presents the opportunity to molecularly define aspects of the acquisition and retention processes and
2. the importance of determing the evolution of mechanisms of learning and memory consolidation.

Preliminary experiments injecting cockroaches with PURO before shock avoidance training in a T-maze (a paradigm similar to ones

frequently used in rodent studies), in doses causing approximately 70 percent protein synthesis inhibition, indicated significant amnesia of the training experience upon testing five hours later. In order to substantiate these results a more detailed study was undertaken to assess not only the effect that pre-training injections of PURO had on invertebrate learning and memory, but, in an effort to define the limits of PURO action in invertebrates and to draw stronger correlations to the vertebrate studies, a series of post-training injection experiments also were performed.

Learning and retention was measured by the number of correct choices during the 20 trials of training and testing respectively, and the number of trials it takes an animal to reach a criterion of five out of six correct responses. The time for an animal to proceed down the T-maze runway (runway time) and the time taken from this point until an actual choice is recorded (choice point time) were other behavioral parameters determined. These responses and measures were useful in resolving the issue of which of the observed behavioral effects of PURO may be attributable to effects on learning, memory, activity, or illness of the animal.

Furtheremore, previous research utilizing the same paradigm as above with pre-training doses of CXM causing over 90 percent protein synthesis inhibition were ineffective in producing retention deficits. These results in conjunction with the preliminary PURO results suggest that the absolute amount of protein synthesis inhibition cannot be the major causal factor in the ability of PURO to produce the observed behavioral amnesia.

A model, based on the vertebrate studies, has been developed that describes antibiotic-induced retention deficits as resulting from other effects of the drugs than their action on overall protein synthesis (Barraco and Stettner, 1976). The model proposes that effects of antibiotics in vertebrates may be tied to effects on neurotransmitter systems. Specifically, the effects of pre-training administration of PURO is hypothesized to be the result of interference with cholinergic transmission. Thus, additional experiments were conducted using scopolamine, a muscarinic blocker, to initially assess the role of cholinergic transmission on invertebrate learning and memory and to determine whether such results can be related to the PURO findings.

The thesis was comprised of the following experiments:

1. A drug toxicity study to determine the maximum allowable dose of PURO and scopolamine that can be injected into each animal and not produce any observable abnormal behavioral effects during the time periods of training and/or testing and up to 4-7 days beyond this period;
2. A profile of the time span of protein synthesis inhibition by PURO to decide when the drug should be injected in separate experiments before and after training so as to be respectively training or testing animals during maximal protein synthesis inhibition;
3. The effects of pre-training administration of PURO on learning and retention of a shock avoidance paradigm;
4. The effects of post-training administration of PURO on retention of a shock avoidance paradigm;

5. **The effects of pre-training administration of scopolamine on learning and retention of a shock avoidance paradigm;**
6. **The effects of post-training administration of scopolamine on retention of a shock avoidance paradigm.**

MATERIALS AND METHODS

Experimental Animals

Adult male cockroaches of the species Periplaneta americana and weighing approximately one gram were used in all experiments. Animals were originally obtained from the U. S. Department of Agriculture, the Canadian Agricultural Institute, Ciba-Gergy Corporation, and the Departments of Entomology at the University of Illinois and Texas A & M University. All new shipments of cockroaches were added to a main colony that varied from several hundred to several thousand animals, both male and female. Animals were given access to dog food and water ad lib, and exposed to light-dark periods of 14 and 10 hours, respectively, at approximately 24°C.

Periodically, adult male cockroaches were removed from the "main" colony and put into a "holding" colony of several dozen male animals, which was located in the laboratory where the experiments were conducted. Otherwise the conditions of the "holding" colony were similar to those described above for the "main" colony. All animals were allowed to acclimate to the "holding" colony for a period of not less than two weeks before use in experimentation.

Determination of Drug Dosages

Puromycin dihydrocholoride (PURO) and scopolamine hydrobromide were obtained from Sigma Company. Toxicity studies were performed before using these drugs. The procedure consisted of injecting different animals (under the abdominal cuticle into the hemolymph near the metathoracic segment) with varying doses of each drug and observing

them for 4-7 days. During this time animals were scrutinized for behavioral changes such as more or less activity, the degree of startle response, the presence or absence of the righting reflex, unusual defecation, or any other behavioral evidence that might indicate physical debilitation.

All drug doses were dissolved in 20 µl of insect Ringer solution (154mM NaCl, 2mM KCL, 0.47mM Na_2HPO_4, 1.8mM $CaCl_2$, 0.2mM KH_2PO_4) and adjusted, if necessary, to a pH of approximately 7.0. Animals (5-12 in each dosage group) were anesthetized with CO_2 before injection. The actual dosage arrived at for use in the behavioral experiments was the largest amount of each drug that could be injected and not produce behavioral abnormalities during the time periods of training and/or testing, or subsequent mortality of any of the animals within a particular group over the 4-7 day observation period.

Measurement of Protein Synthesis Inhibition by PURO

The dosage arrived at for PURO in the preceding section was used to measure the drug's time course of protein synthesis inhibition. This information was critical in determining at which times animals were to be injected before or after training so as to be either training or testing during maximum protein synthesis inhibition of cockroach central nervous system.

The incorporation of ^{14}C-leucine into protein was used to estimate the degree of protein synthesis inhibition. Animals were injected with either PURO or insect Ringers solution (saline). At various intervals after injection of the drug or vehicle, 10 µl of ^{14}C-leucine (New England Nuclear Corporation; 315 mCi/mM) was injected. (For the one-hour

interval the label and PURO were injected simultaneously.) One hour after the leucine injection, the brain and three thoracic ganglia were dissected out and immediately placed in ice cold insect Ringer solution. Incorporation of labelled leucine into ganglionic and brain protein was determined by the method of Kerkut, et al., (1970) and later modified for this preparation by Lovell (1975). The tissue of five animals was used in each group. After dissection, the tissue was homogenized (using a hand-operated glass homogenizer) in 1 ml of cold saline. The homogenate was transferred to a centrifuge tube and the homogenizer was rinsed with 1 ml of saline. From this quantity 0.5 ml was removed to determine the amount of protein in each sample by the method of Lowry, et al., (1951) (see Appendix A). Three ml of 10 percent trichloracetic acid (TCA) solution was then added to the remaining sample in the centrifuge tube. This mixture was thoroughly agitated for 30 seconds and centrifuged at 3000 g for ten minutes. One ml of the supernatant was removed, mixed with 15 ml of PCS scintillation fluid (Amersham/Searle Corporation), and counted for 20 minutes on a Packard Tri-Carb Liquid Scintillation Spectrometer, and corrected for quenching. The pellet was resuspended in 2 ml of chloroform/methanol (1:1) and centrifuged at 3000 g for ten minutes. To the resulting pellet, 1 ml of 1N NaOH was added and the mixture was put in a water bath at 100°C for ten minutes. The ensuring solution was cooled and centrifuged to remove undissolved material. The supernatant was removed and counted for 20 minutes. Inhibition of protein synthesis was calculated according to the method of Barondes and Cohen (1967b). The percentage inhibition was calculated at $1 - (R_{PURO}/R_{Sal}) \times 100$, where R is the ratio of counts

per minute (cpm) per mg of protein present of the TCA percipitable fraction to cpm per mg of protein of the TCA soluble fraction. Since the TCA percipitable fraction contains the labelled leucine which has been incorporated into protein, a reduction in the cpm of the TCA precipitable fraction in the PURO treated tissue as compared to control tissue indicates a reduction in the amount of protein synthesis occurring between the ^{14}C-leucine injection and removal of the tissue.

T-Maze Training

Apparatus

A diagram of the T-maze is shown in Figure 1. The maze is constructed of Plexiglas, and consists of a start box (S) (5.0 cm X 3.2 cm X 3.8 cm), a runway (R) (15.5 cm X 3.2 cm X 3.8 cm), two choice arms (7.5 cm X 3.2 cm X 3.8 cm), and two goal boxes (G) (5.5 cm X 3.2 cm X 3.8 cm). The goal boxes can be moved from the start box entrance to the choice arm exit. The walls of the runway and choice arms are opaque and the ceiling is transparent. The two goal boxes which contain plungers are fixed at 90° angles to the choice arms, are totally opaque. They provide small dark enclosures which act as a reward for the cockroaches, which are known to prefer dark to light. Manually operated guillotine doors are located at the entrance to each goal box, the entrance and exit of the start box, the end of the runway, and at the entrance of each choice arm. The shock grids on the floor of the choice arms were made by photoetching copper film on a glass epoxy backing to form 2 mm wide strips separated by 0.8 mm. The source of shock is a 60 Hz AC current reduced to approximately 10 V with a

Figure 1. Schematic representation of the T-maze used for shock avoidance training and testing of cockroaches. The dotted lines located at the entrance to the start box, runway, and at points labeled by letters A, B, and C 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. Abbreviations used: G, goal box; P, plunger; S, start box; R, runway; A, B, and C, sliding doors.

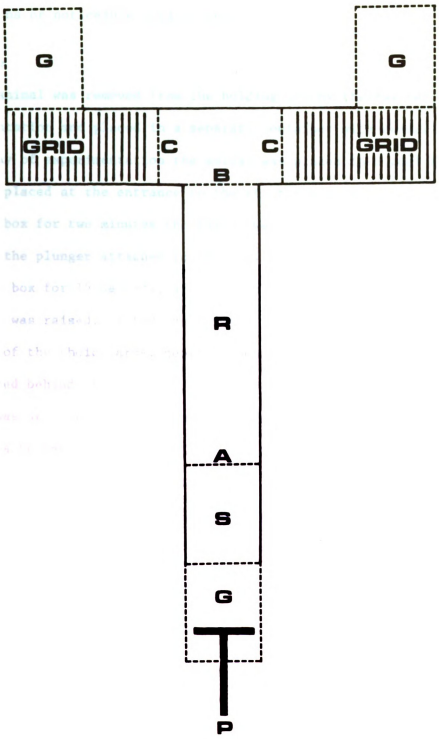

Figure 1

variable transformer. The amplitude was set so that the shock caused immediate escape from the incorrect choice arm, but did not produce convulsions or noticeable erratic behavior.

Procedure

An animal was removed from the holding colony the day before experimentation and placed in a separate container with access to water. On the day of experimentation the animal was placed in a goal box which was then placed at the entrance to the start box. After remaining in the goal box for two minutes the animal was gently pushed into the start box with the plunger attached to the goal box. The animal remained in the start box for 15 seconds, after which the door to the runway (A in Figure 1) was raised. After the animal proceeded down the runway and into one of the choice arms, door B, then door C on the appropriate side, were closed behind the animal. If the cockroach did not leave the start box, or was stationary in the runway or the choice point for more than 20 seconds he was gently prodded by placing a light brush stroke medially to the inferior part of the wings.

The training procedure consisted of an initial "free trial" in which no shock was given, and 20 subsequent trials in which shock was administered in one choice arm. The animal was trained to turn opposite to the direction it chose on the first trial (after the free trial), so that the animal always made an incorrect choice and received a shock on the first trial. This was done in order to insure against any direction preference the animal might display. A choice was recorded when the animal placed two legs upon a grid surface (see Figure 2). If the

Figure 2. Photograph of cockroach in various positions in T-maze. Top left: shows an animal in the runway. Top right: depicts an animal at the choice point, marking the beginning of choice point time. Bottom: demonstrates an animal placing two legs upon an electrical grid, thus designating a choice and the end of the choice point time.

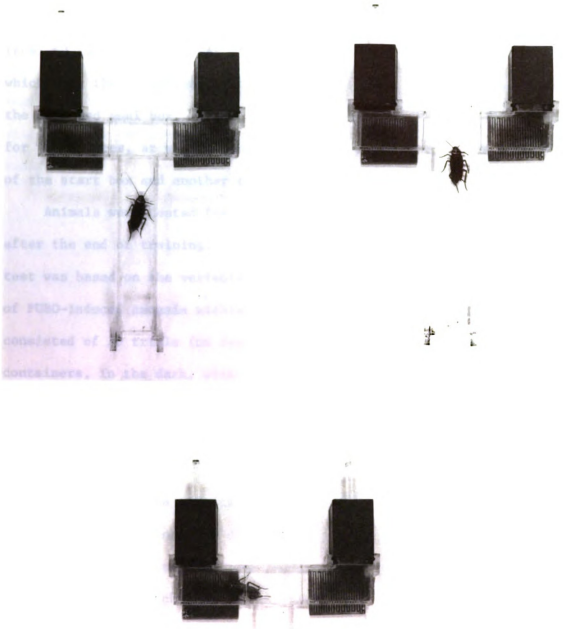

Figure 2

animal placed two legs onto the incorrect choice arm grid a shock was received causing the animal to immediately leave that choice arm, in which case the animal was allowed to enter the correct choice arm and the attached goal box. After entering the goal box the animal remained for two minutes, at which time the goal box was placed at the entrance of the start box and another trial began.

Animals were tested for retention of the training five hours after the end of training. The time interval selected for the retention test was based on the vertebrate literature which suggested the presence of PURO-induced amnesia within this time period. The testing procedure consisted of 20 trials (no free trial). Animals were kept in individual containers, in the dark, with access to water, between training and testing.

Four behavioral parameters were recorded during training and testing:

1. Runway time--the time taken by the animal to proceed down the runway to the location of door B;
2. Choice point time--the time taken by the animal to proceed from door B until a choice was recorded by the animal placing two legs upon a choice arm grid;
3. Choice behavior--the direction turned;
4. Trials to 5/6--the number of trials taken by the animal until he responded correctly in 5 out of any 6 trials.

For analysis, the data was divided into trials of ten during training and testing. Previous work using the same paradigm (Lovell, 1975)

indicated that this division was of sufficient magnitude to indicate both acquisition and retention of the training. Statistical comparisons were done by means of the student's two-tail t-test with the level of significance set at a P value of 0.05 or less (unless otherwise stated). The experimentor was unaware as to what treatment an animal received.

RESULTS AND DISCUSSION

Determination of Drug Dosages

The largest dosage of puromycin (PURO) that conformed to the criteria outlined in the Methods section was 300 µg. Dosages of 400 µg and greater resulted in partial to total lethality of the experimental groups. The actual effect that this dosage of PURO has on synthesis of central nervous system proteins and the injection times needed to ensure maximum effect of the drug was determined by the results of the experiments detailed in the next section.

The dosage of scopolamine arrived at through similar toxicity studies was 500 µg. Dosages of 750 µg and greater resulted in death of the animals or an unacceptable dimunition of righting and/or startle reflexes one or more hours following injection. Evidence that this drug dosage was affecting the central nervous system was demonstrated by: (1) animals frequently displaying "twilight-like sleep" characteristics (Goodman and Gilman, 1975) when injected with varying amounts of the drug, and (2) motor impairment that could not be attributed to peripheral cholinergic interference since there are no cholinergic skelatal neuromuscular junctions in arthropods (Callec, 1974).

CNS Protein Synthesis Inhibition by Puromycin

Figure 3 shows the amount of protein synthesis inhibition produced by 300 µg of PURO at several intervals after injection. It also depicts the amount of inhibition caused by 250 µg of cycloheximide (CXM). This CXM dosage was used by Lovell (1975) in training and testing cockroaches in the exact same paradigm. The comparative data demonstrates that the

dosage of CXM, that produced no retention deficits, caused substantially greater and faster protein synthesis inhibition of the central nervous system than the dosage of PURO utilized in this study. Cycloheximide produced close to 95 percent inhibition for at least 1-7 hours after injection, while, PURO produced a maximum of about 70 percent inhibition that took three hours to fully develop. These findings are consistent with comparative inhibition data for CXM and PURO in fish (Brink, et al., 1966), rodents (Flexner, et al., 1965), and pigeons (Stettner, et al., 1977).

Based on these results PURO was injected three hours before training for the pre-training experiments and three hours before testing (two hours after training) for the post-training experiments so as to maximize any possible protein synthesis inhibition effects that PURO may have upon learning and retention mechanisms.

The nine hour interval after PURO injection in Figure 3 corresponds to the amounts of inhibition at the time of retention testing for the pre-training injection group (i.e., injection three hours prior to training + one hour for training + five hour delay before testing). The two hour and four hour interval of the PURO inhibition profile (not graphed) show a correspondant increase and then decrease of inhibition to and from the three hour interval and were measured at 67 and 50 percent, respectively.

Pre-Training Puromycin (PURO) Administration

Choice Behavior

Figure 4 and 5 and Table 1 detail the effects of pre-training administration of PURO and saline on acquisition and retention of the

Figure 3. Protein synthesis inhibition profile at various times after injection of puromycin (PURO) or cycloheximide (CXM). Each point was obtained by measuring incorporation of ^{14}C-leucine into protein for the pooled tissue of five animals. The results for the one hour and seven hour CXM groups estimate the degree or protein synthesis inhibition present during training and testing (five hours after the end of training), respectively, of the CXM-injected animals in the behavioral experiments performed by Lovell (1975), using the exact same paradigm as detailed for the PURO experiments in this dissertation. The results for the three hour and nine hour PURO groups estimate protein synthesis inhibition during training and testing for the pre-training PURO-injected animals. The three hour group would also represent the degree of protein synthesis inhibition during testing of the post-training PURO-injected animals. The two hour and four hour interval of the PURO inhibition profile (not graphed) were measured at 67 and 50 percent, respectively. All points are measured to within an accuracy of ± 10 percent.

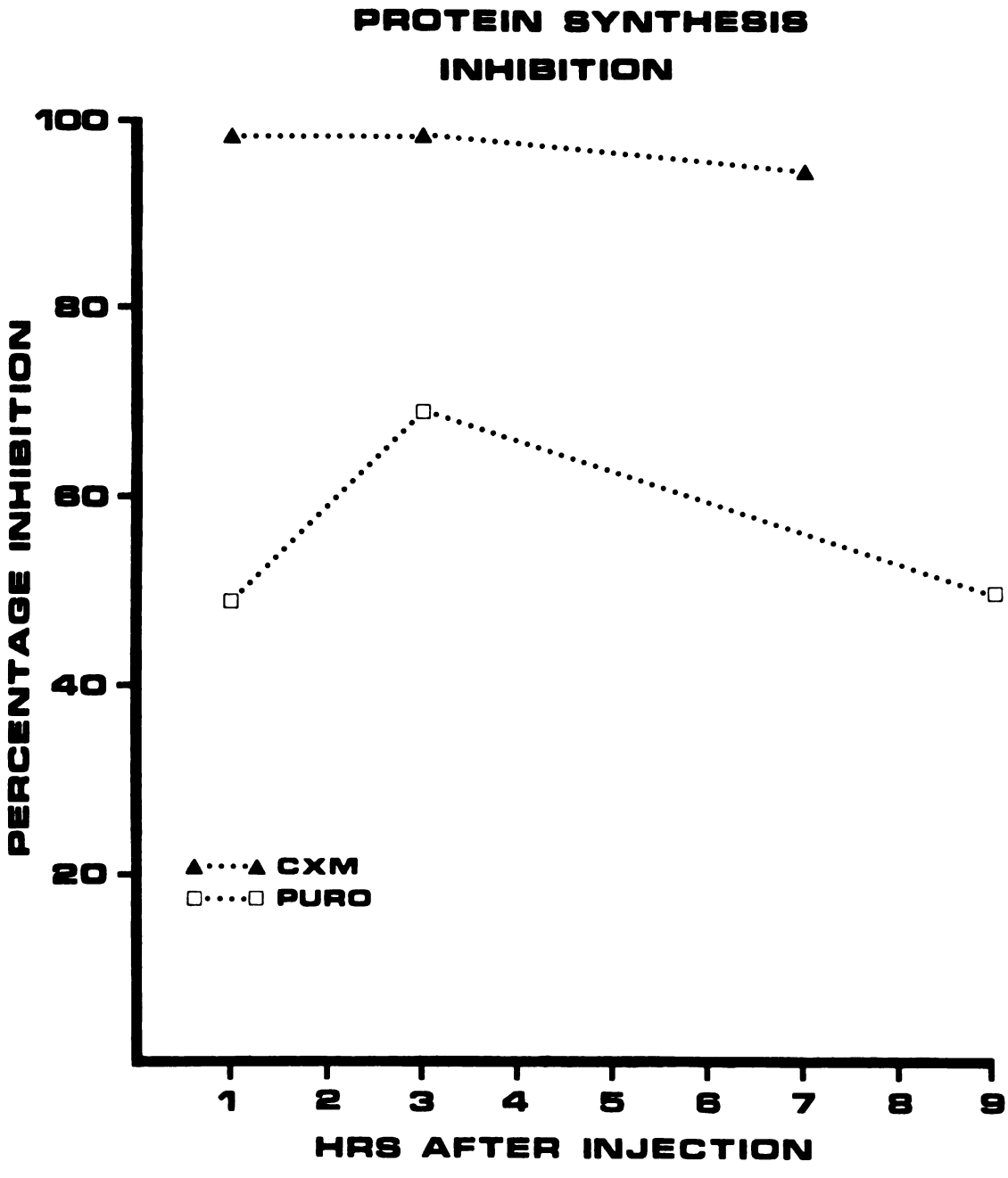
PROTEIN SYNTHESIS
INHIBITION
100
80
60
40
20
PERCENTAGE INHIBITION
CXM
PURO
1
2
3
4
5
6
7
8
9
HRS AFTER INJECTION

Figure 3

Figure 4. Mean number of correct turns during training and testing for animals injected before shock avoidance training with puromycin (PURO) (n=31) or saline (n=23). During testing trails 1-10 of the PURO animals made significantly fewer correct turns than the saline group did during testing trials 1-10 and than the saline and PURO animals did during training trials 11-20. Standard errors of all points are given in Table 1.

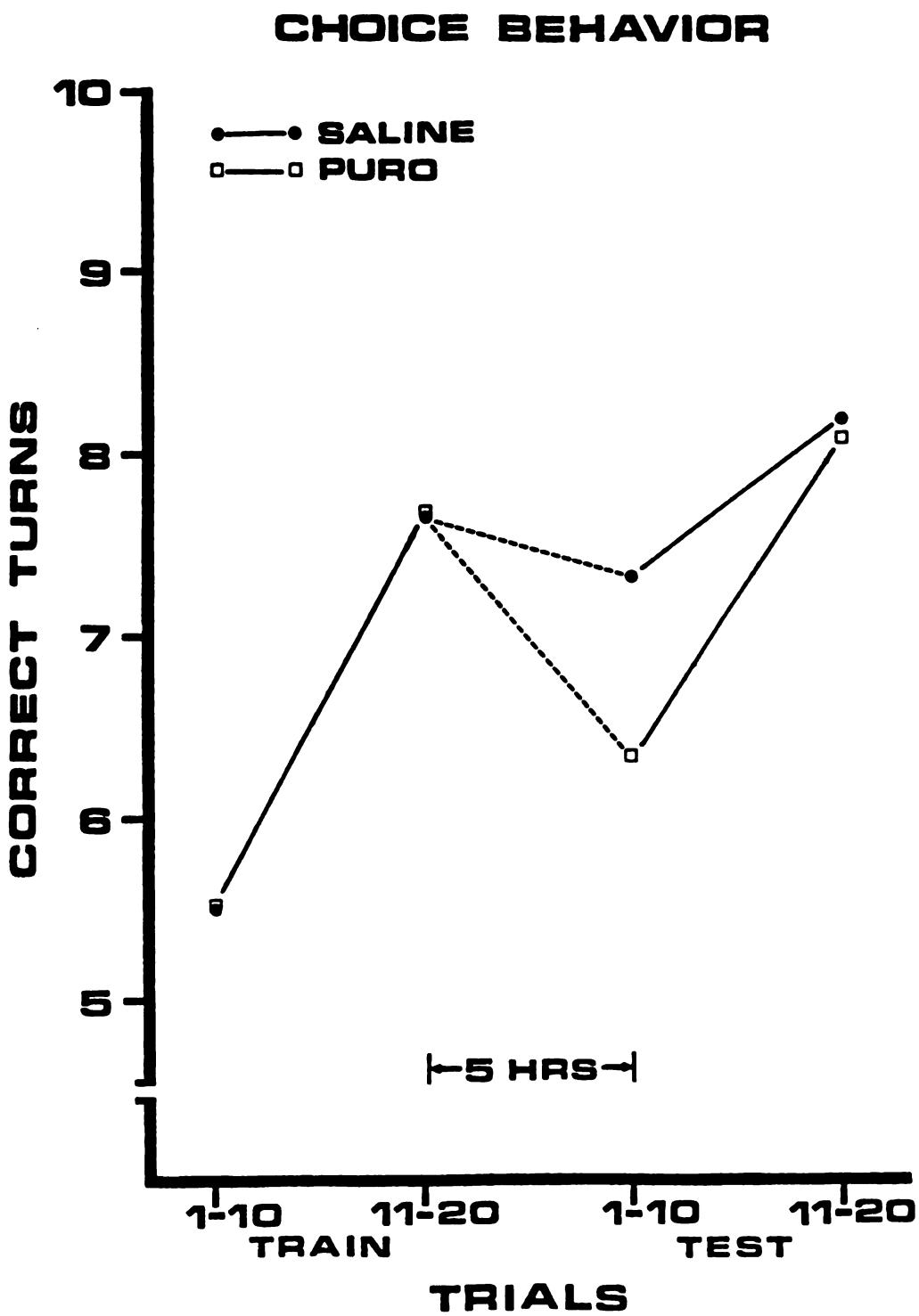
CHOICE BEHAVIOR
SALINE
PURO
CORRECT TURNS
10
9
8
7
6
5
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 4

Figure 5. Mean number of trials to a criterion of five correct turns out of any six trials during training and testing for animals injected before shock avoidance training with either puromycin (PURO) (n=31) or saline (n=23). During testing the PURO animals took significantly more trials to criterion than the saline group, thereby indicating a loss of retention from training to testing.

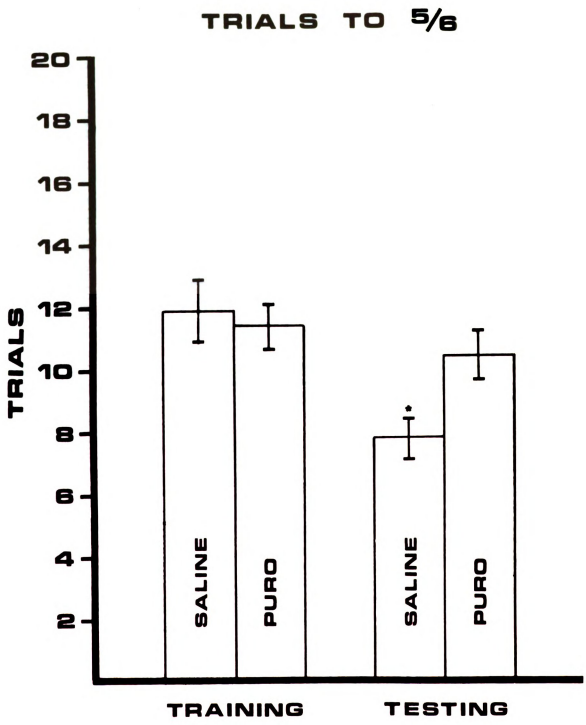
TRIALS TO 5/6
TRIALS
20
18
16
14
12
10
8
6
4
2
SALINE
PURO
SALINE
PURO
*
TRAINING
TESTING

Figure 5

Table 1. Comparison of mean number of correct turns and trials to criterion during training and testing of animals injected with puromycin (PURO) or saline three hours before (Pre-PURO) or two hours after (Post-PURO) shock avoidance training, and a group of animals given no injection and exposed to the paradigm without shock applied. All animals were given 20 training trials followed five hours later by 20 testing trials.

	TRAINING				TESTING			
	Correct Turns			Trials to	Correct Turns			Trials to
Treatment (n)	1-10	11-20	% improvement	5/6	1-10	11-20	% loss	5/6
Saline (23)	5.52 ± 0.34[a]	7.65 ± 0.39	39[b]	11.96 ± 0.98[c]	7.35 ± 0.25	8.22 ± 0.17	14[d]	7.83 ± 0.60
Pre-PURO (31)	5.55 ± 0.25	7.70 ± 0.24	39	11.39 ± 0.69	6.35 ± 0.29	8.13 ± 0.29	61	10.42 ± 0.76
Post-PURO (10)	5.10 ± 0.40	7.30 ± 0.45	43	13.70 ± 1.54	6.40 ± 0.45	8.00 ± 0.33	41	9.60 ± 1.21
No-Shock (6)	4.50 ± 1.17	4.67 ± 1.23	3	15.17 ± 2.24	4.50 ± 1.43	5.33 ± 1.33	100	13.83 ± 3.38

[a] Mean ± S.E.M.

[b] % improvement is calculated by subtracting the mean correct turns for training trials 1-10 from mean correct turns for training trials 11-20 and dividing by the mean correct turns training trials 1-10

[c] refers to the number of trials that the animal took to reach a criterion of 5 correct turns out of any 6 trials

[d] %loss is calculated by subtracting the mean correct turns for testing trials 1-10 from the mean correct turns for training trials 11-20 and dividing by the difference in the mean number of correct turns of training trials 11-20 and training trials 1-10

correct turn response. Comparison of the mean number of correct turns for training trials 1-10 with training trials 11-20 demonstrates that both groups of animals learn the maze equally well. There being a statistically significant improvement of 39 percent between the first and second ten trials of training for both groups. Thus, both groups exhibit shorter-term memory and PURO has no effect on acquisition of the avoidance training.

Comparison of the means number of correct turns between training trials 11-20 and testing trials 1-10 can be used to measure the level of retention of the training experience five hours later. This comparison demonstrates a statistically significant retention of the avoidance training for the saline group. These animals only display a 14 percent loss in retention of the correct turn response; i.e., the saline group retains 86 percent of this training parameter. By contrast, the group injected with PURO exhibits a statistically significant retention loss of the correct turn response. For this group, the difference between the mean number of correct turns from training trials 11-20 to testing trials 1-10 represents a 61 percent loss or a retention of only 39 percent. A comparison of the difference in the mean number of correct turns for testing trials 1-10 for the PURO and saline groups also is statistically significant, further confirming an amnesia for the PURO animals (Figure 4, Table 1).

Additional substantiation of this amnesic effect of the correct turn response is provided by the mean number of trials to criterion data (Figure 5, Table 1). These results show that both saline and PURO animals reach criterion at approximately identical times (i.e., 11-12th trial). However, during testing the group injected with

PURO requires a statistically greater number of trials to reach criterion than does the saline group. Statistically approached in another way, the saline animals reach criterion significantly faster during testing than training, demonstrating a retention of the training experience. The PURO group does not.

There are immediate observations concerning the cause and nature of the amnesia. One is that the overall amount of protein synthesis inhibition does not appear to be the major causal factor in the induced retention deficit. This statement is based on the fact that Lovell (1975), utilizing the same paradigm and pre-training injections of CXM in doses causing substantially more protein synthesis inhibition (i.e., over 90 percent) than the doses of PURO used in the present study, found no indication of reduced retention when animals were tested five minutes, one hour, five hours, and 22 hours after training.

However, it may be possible that PURO is selectively inhibiting the formation of proteins or peptides crucial for longer-term memory formation which are not affected by CXM. Protein synthesis is a multi-step process, and CXM and PURO do inhibit at different sites in the overall process. But in either case the net result is the same, that is, the overall process of protein synthesis is blocked, no matter at which step the inhibition occurs. Hence, it may be difficult to envision how different classes of proteins may selectively slip past one or either of these inhibitors. Nevertheless, there are data that purport to show differential effects of PURO and CXM on the protein synthetic machinery. For example, Morgan and Austin (1968) show that PURO inhibits both cytoplasmic and mitochondrial synthesis maximally

while CXM inhibits cytoplasmic protein synthesis only. Further, MacInnes and Luttges (1972, 1973) have shown that PURO produces disaggregation in a polyribsomal population which is distinct from the polyribsomal population affected by CXM. Also, Wilson (1971), working with the gastropod mollusk, Aplysia, noted a selective decrease in the amount of leucine - ^{3}H incorporated into higher molecular weight classes of proteins in single identifiable neurons incubated in PURO, possibly signifying a selective inhibition of higher molecular weight proteins. Finally, Rothman and Strumwasser (1976) found a pronounced decrease in the incorporation of leucine - ^{3}H into proteins above 75,000 daltons in Aplysia eyes treated with PURO. The authors noted a "less steep decrease in CXM-treated eyes" despite the fact that both drugs caused the same overall amount of protein synthesis inhibition (i.e., 50 percent). When these authors tested doses of PURO causing over 80 percent protein synthesis inhibition they found "leucine - ^{3}H incorporation into proteins was strongly inhibited at molecular weights above 12,000 daltons and that the inhibition of leucine - ^{3}H incorporation increased with the apparent molecular weight of the labeled material."

No research has yet been performed that attempts to explain the disparity of effects of PURO and CXM on the basis of selective protein synthesis inhibition. It would appear that such work is warranted and would be largely welcomes by proponents of the theory that the amnesic effects of PURO and CXM are due, at least in part, to pertubations of the protein synthetic machinery of the central nervous system.

In addition to ruling out the absolute amount of protein synthesis inhibition as the cause for the observed amnesia, it also appears that

illness of the animal due to unobservable drug toxicity can also be eliminated as a causative factor. This is supported by two facts: First, the animals injected with PURO learn the avoidance training as well as the saline animals. Similarly, the PURO animals show excellent re-learning of the maze during testing, exhibiting a 28 percent improvement in the mean number of correct turns between testing trials 1-10 and testing trials 11-20 (Figure 4). This type of behavior would not be expected of an animal undergoing a drug-induced performance deficit. Secondly, the runway times (see below) for the PURO animals are as fast or faster than the saline animals. If the animals of this group were debilitated it would be anticipated that movement down the runway would be impaired or slowed.

Runway Time

Figure 6 shows the mean amount of time taken for the two groups of animals to proceed from the start box to the choice point (runway time). This time was originally measured to determine if the animals were being incapacitated by PURO. However, closer analysis has made it a much more interesting parameter.

As mentioned above, the mean runway times indicate that animals injected with PURO were not physically impaired since they proceeded down the runway as fast or faster than the control animals. In addition, both groups of animals show the same change in behavior during training, with a progressive increase in mean runway time with succeeding trials. This increase is statistically significant for both groups.

Figure 6 Mean runway times during training and testing for animals injected before shock avoidance training with either puromycin (PURO) (n=31) or saline (n=23). Both groups of animals show a significant increase in the time taken in the runway between training trials 1-10 and training trials 11-20. In addition, both groups show retention of this behavioral modification in that neither group showed a statistically significant drop in time between training trials 11-20 and testing trials 1-10. During training trials 11-20 the saline group exhibited significantly longer (slower) runway times than the PURO animals. All other points are statistically indistinguishable. Standard errors of all points are given in Table 2.

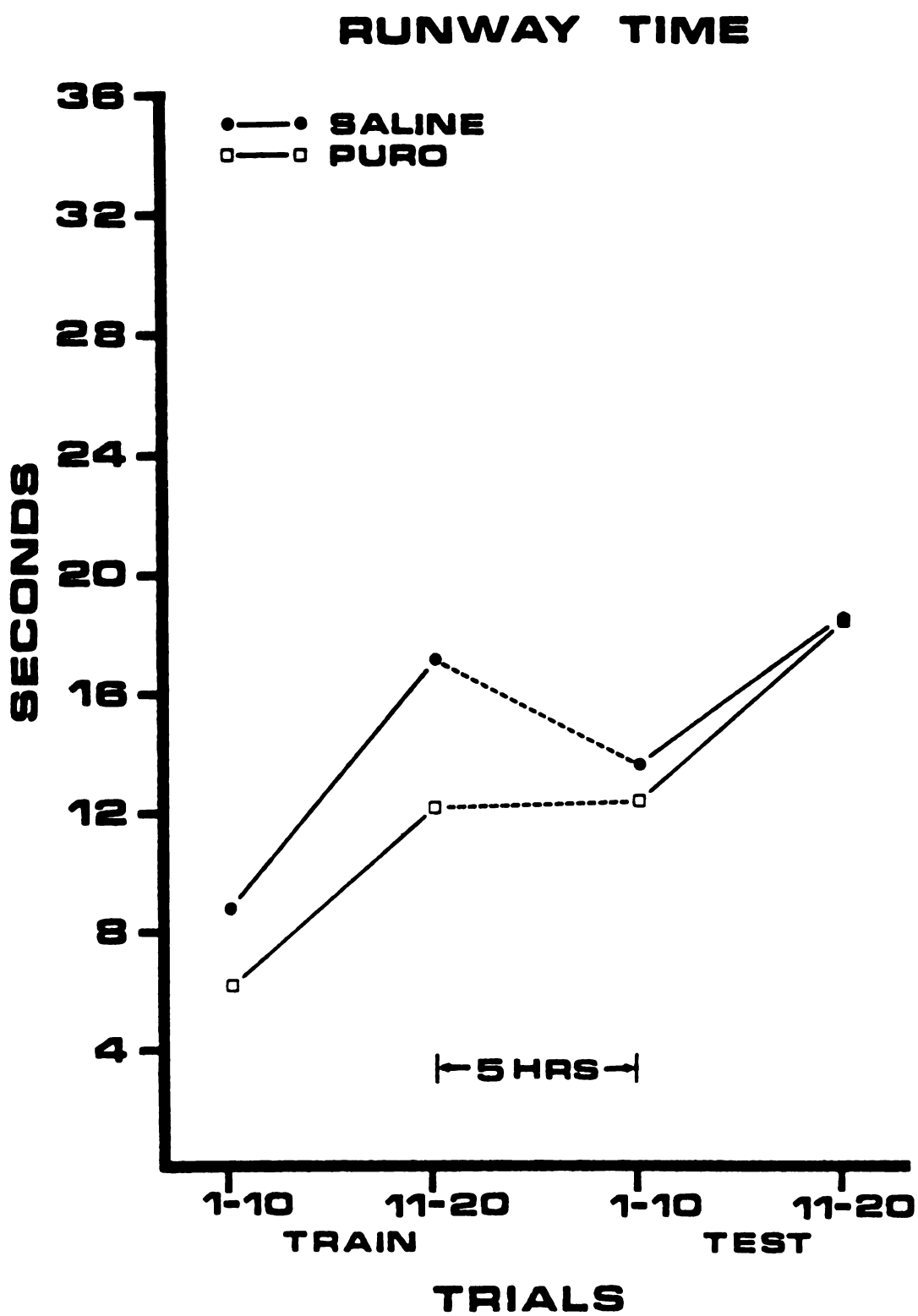
RUNWAY TIME
SALINE
PURO
36
32
28
24
20
16
12
8
4
SECONDS
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 6

Table 2. Comparison of mean runway and choice point times during training and testing for animals injected with puromycin (PURO) or saline three hours before or two hours after shock avoidance training, and a group of animals given no injection and subjected to the paradigm without shock. All animals were given 20 training trials followed five hours later by 20 testing trials.

MEAN RUNWAY TIMES

Treatment (n)	Training Trials 1-10	Training Trials 11-20	Testing Trials 1-10	Testing Trials 11-20
Saline (23)	8.7 ± 1.3 *	17.1 ± 2.1	13.8 ± 1.8	18.6 ± 2.1
Pre-PURO (31)	6.4 ± 0.9	12.2 ± 1.4	12.6 ± 1.6	18.6 ± 2.6
Post-PURO (10)	7.6 ± 1.8	16.2 ± 3.8	4.9 ± 1.4	11.8 ± 3.0
No Shock (6)	9.5 ± 2.0	23.0 ± 3.2	17.7 ± 1.4	33.2 ± 1.9

MEAN CHOICE POINT TIMES

Treatment (n)	Training Trials 1-10	Training Trials 11-20	Testing Trials 1-10	Testing Trials 11-20
Saline (12)	11.3 ± 3.8	13.7 ± 3.9	14.7 ± 3.2	17.0 ± 4.3
Pre-PURO (21)	19.5 ± 1.8	27.2 ± 3.1	30.5 ± 3.9	25.5 ± 4.2
Post-PURO (10)	40.3 ± 5.5	41.4 ± 5.4	24.0 ± 3.7	28.3 ± 5.9
No Shock (6)	7.3 ± 3.1	12.7 ± 3.0	17.3 ± 3.4	18.6 ± 4.0

*Mean ± S.E.

One possible explanation for the increased runway time could be general habituation, an elementary form of learning. It is a well-known phenomenon that animals that are placed in a novel environment will exhibit exploratory or curiosity behavior. This would initially be reflected by increased activity, thus faster runway times. As the animal becomes acclimated to the situation, interest wanes and the activity levels decline, resulting in longer runway times.

Another possible explanation could be that the progressive increase in runway time may reflect associative learning. That is, the animal is learning to associate the runway with the possibility of receiving a shock, and is showing avoidance behavior by moving more slowly down the runway.

In order to determine if the progressive increase in runway time resulted from habituation or reflected an association of the runway with the possibility of receiving shock, a group of non-injected animals were given trials in the maze with no shock administered. In all respects the "training" and "testing" procedures were identical to those given shock. Although there was not an actual correct or incorrect choice a choice was recorded and labeled correct or incorrect based upon the criteria mentioned in the Methods section for animals receiving a shock. If the increase in runway time seen in Figure 6 represents general habituation, then qualitatively similar changes would be expected in the runway behavior of the "no shock" group (i.e., increased runway time with succeeding trials). If, however, the increase in runway time indicates primarily an active avoidance response than the "no shock" group would not be expected to show an increase in runway time with succeeding trials in the maze.

The results, shown in Figures 7 and 8 and Tables 1 and 2, demonstrate that while no preferred choice was seen (Figure 7), as would be expected without administration of a shock, the "no shock" group behaved similarly to the other groups in showing a marked increase in mean runway time during training (Figure 8). These data suggest that the increase in runway time observed in the groups receiving shock was due to habituation in the maze and cannot be attributed to an association of the runway with the possibility of receiving shock. In fact, the animals receiving the shock showed less habituation perhaps signifying a general state of central nervous system arousal due to the shock received by these animals.

Testing results show retention of this habituation behavior for the saline, PURO, and "no shock" groups. A comparison of the mean runway times for training trials 11-20 with testing trials 1-10 failed to show a statistically significant drop for any group (Figure 8). The inability of PURO to affect cockroach maze habituation has been supported in other work by Lovell and Eisenstein (unpublished). These authors showed that neither acquisition nor retention of habituation of arm entries in a Y-maze by cockroaches (n=6) were influenced by the same dose of PURO used in the present experiments. Therefore, while PURO produces a retention deficit of the choice behavior response (representing one form of learning and memory) it does not alter retention of runway habituation (representing another form of learning and memory). Thus, PURO apparently does not obliterate all of the different memories which may occur in a given shock avoidance training situation.

Figure 7. Mean number of correct turns during training and testing for animals injected before shock avoidance training with puromycin (PURO) (n=31) or saline (n=23) and a group of animals (n=6) given no injection and exposed to the paradigm without any shock applied. As would be expected, the "no shock" group did not show improvement with succeeding trials in the maze, strengthening the position that the paradigm and the apparatus are effective in training cockroaches to avoid an electrical shock. Standard errors of all points are given in Table 1.

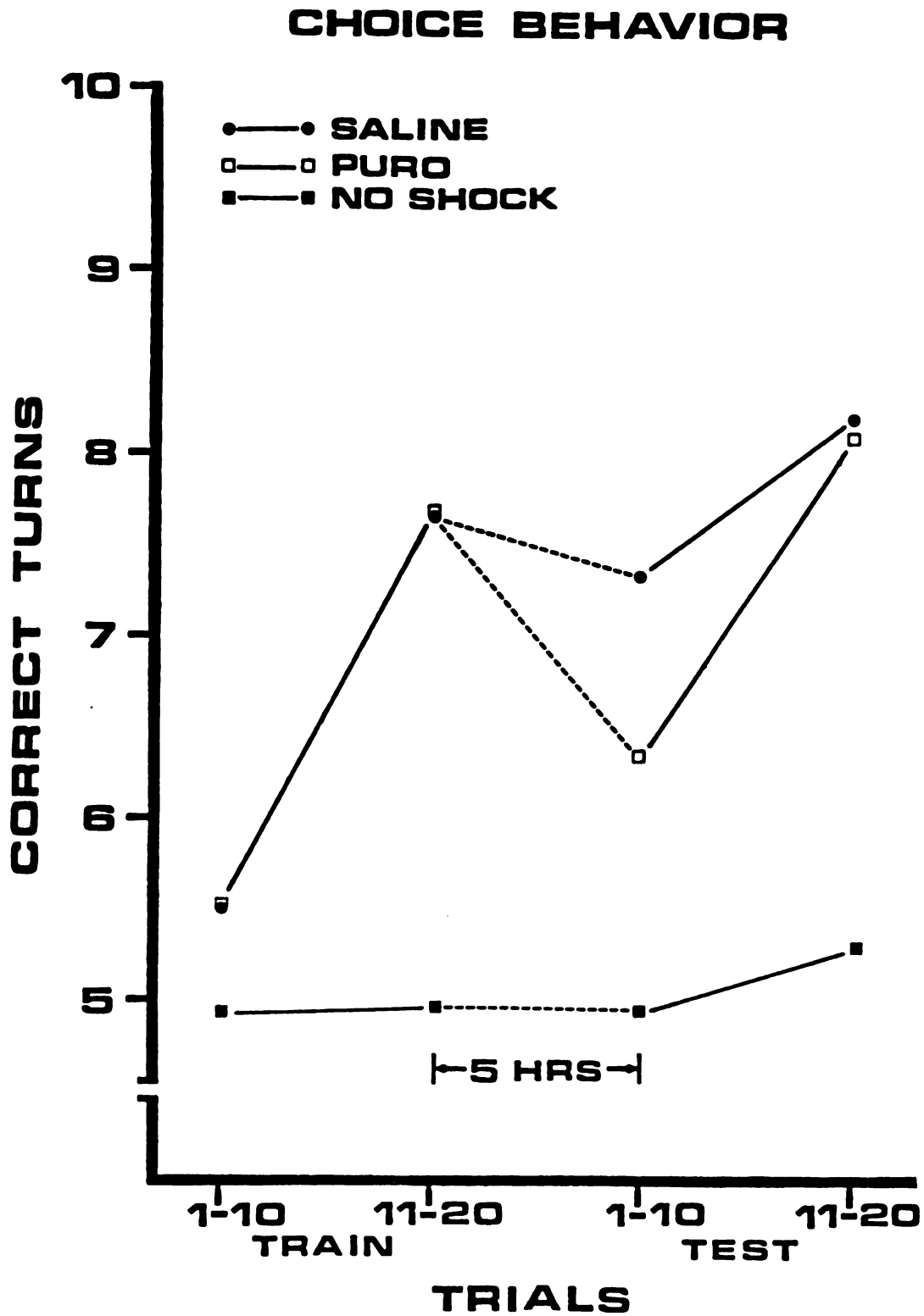
CHOICE BEHAVIOR
SALINE
PURO
NO SHOCK
CORRECT TURNS
10
9
8
7
6
5
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 7

Figure 8. Mean runway time during training and testing for animals injected before shock avoidance training with puromycin (PURO) (n=31) or saline (n=23) and a group of animals (n=6) given no injection and exposed to the paradigm for 20 trials of training and testing without any shock applied. The "no shock" group exhibited the same behavior as the PURO and saline animals, showing a significant increase in the time taken in the runway with succeeding trials in the maze. In addition, there is no significant decrement in any group between training trials 11-20 and testing trials 1-10, indicating significant retention of this behavioral modification. The "no shock" animals exhibit significantly longer (slower) runway times than the PURO animals during training trials 11-20 and testing trials 1-10, and over both saline and PURO groups during testing trials 11-20. Standard errors of all points are given in Table 2.

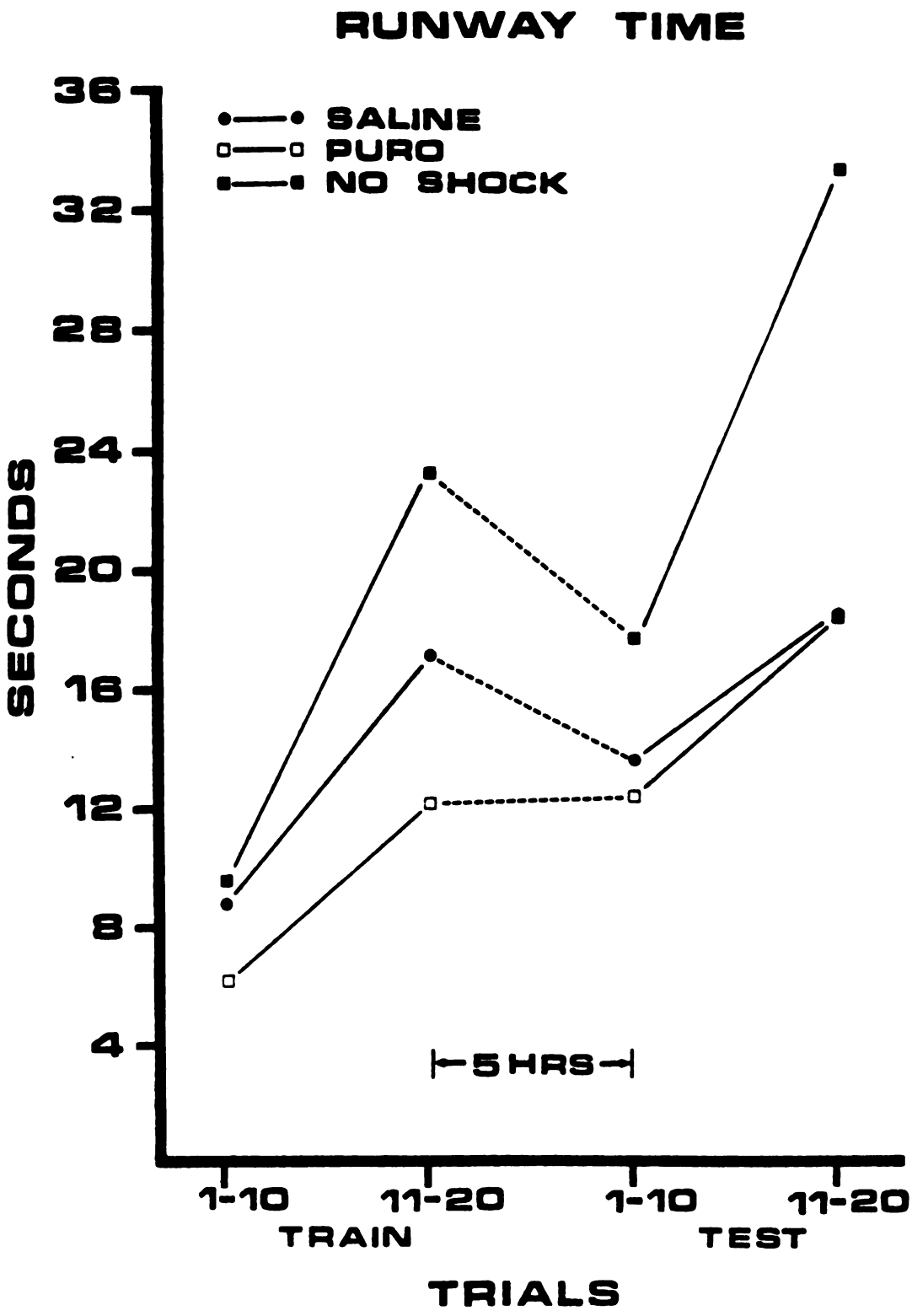
RUNWAY TIME
SALINE
PURO
NO SHOCK
36
32
28
24
20
16
12
8
4
SECONDS
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 8

A differential effect of PURO on the different memories that accompany a training situation also was observed by Schoel and Agranoff (1972) in goldfish. They found that PURO injected just before or immediately after a training session in a shuttle box did not block retention of conditioned cardiac deceleration. Although it did produce a retention deficit of the shock-avoidance response. Schoel and Agranoff hypothesized that the more neurally complex a learned task is (i.e., more synapses), the more susceptible it is to interference by amnesic agents, such as PURO. To quote their discussion, "If an agent such as puromycin reduced the efficacy of every synapses by 1 percent, then in a series of polysynaptic pathway of 10 synapses, the net reduction of signal would be 10 percent (0.9^{10}). A 1 percent reduction in a network of 100 synapses would result in a 65 percent signal reduction (0.9^{100}). From a more general standpoint, we can visualize a complex neural signal that must be distinguished or a highly coordinated motor response which must be organized, and in which a given amount of behavioral, electrical, or chemical 'noise' would generate more interference than in a less complex process."

According to this rationale memory disrupting agents would be expected to have a much more profound effect on behaviors that require more neural events, regardless if this interference is a blockage of a chemical process necessary for the formation of memory or an alteration of the chemical and/or electrical events leading to its formation. Thus, they suggest avoidance conditioning is more susceptible to an amnesic agent than is conditioning of an automatic response simply because of its more complex neural circuitry.

A differential effect of amnesic agents on the different memories accompanying a learning situation in mammals also has been observed. Hine and Paolino (1970) noted electroconvulsive shock was more disruptive of the retention of somatically mediated than autonomically mediated responses in the rat.

These results raise the possibility that in the cockroach the act of making a choice to turn right or left may be mediated at a higher level of the central nervous system and involve a more complex circuit than that mediating runway habituation behavior. Further, these neural circuits may differ in their susceptibility to the action of PURO. Generalizing these results further, one might hypothesize that the effect of PURO in producing amnesia across phyla is determined primarily by the complexity of the neural circuitry involved in a given act rather than by whether the learning is somatically or autonomically mediated or whether it is produced by Pavlovian or instrumental conditioning procedures.

Choice Point Time

Choice point time, the time taken by an animal to proceed from the end of the runway until a choice was recorded by an animal placing two legs upon a grid surface in an arm of the maze, for PURO and saline groups is shown in Figure 9 and Table 2. The results may be summarized as the following: (1) PURO animals exhibit a significant increase in the mean amount of time taken to make a choice between training trials 1-10 and 11-20 and display significant retention of this behavioral change during testing; (2) the saline animals did not show a

Figure 9. Mean choice point times during training and testing for animals injected before shock avoidance training with puromycin (PURO) (n=21) or saline (n=12). PURO animals (1) exhibit a significant increase in the amount of time taken to make a choice between training trials 1-10 and 11-20 and retention of this behavioral change, (2) saline animals do not show a significant increase in choice point time during training, (3) during training trials 11-20 and testing trials 1-10 the PURO group took a statistically longer time to make its choice than the saline group, (4) the PURO group took a statistically longer time moving from the end of the runway to a choice than in traversing the runway itself, despite the former distance being approximately one-third of the latter. The n's differ from Figure 8 because choice point time was not measured in the first 10 PURO and 11 saline animals. Standard errors of all choice point times are given in Table 2.

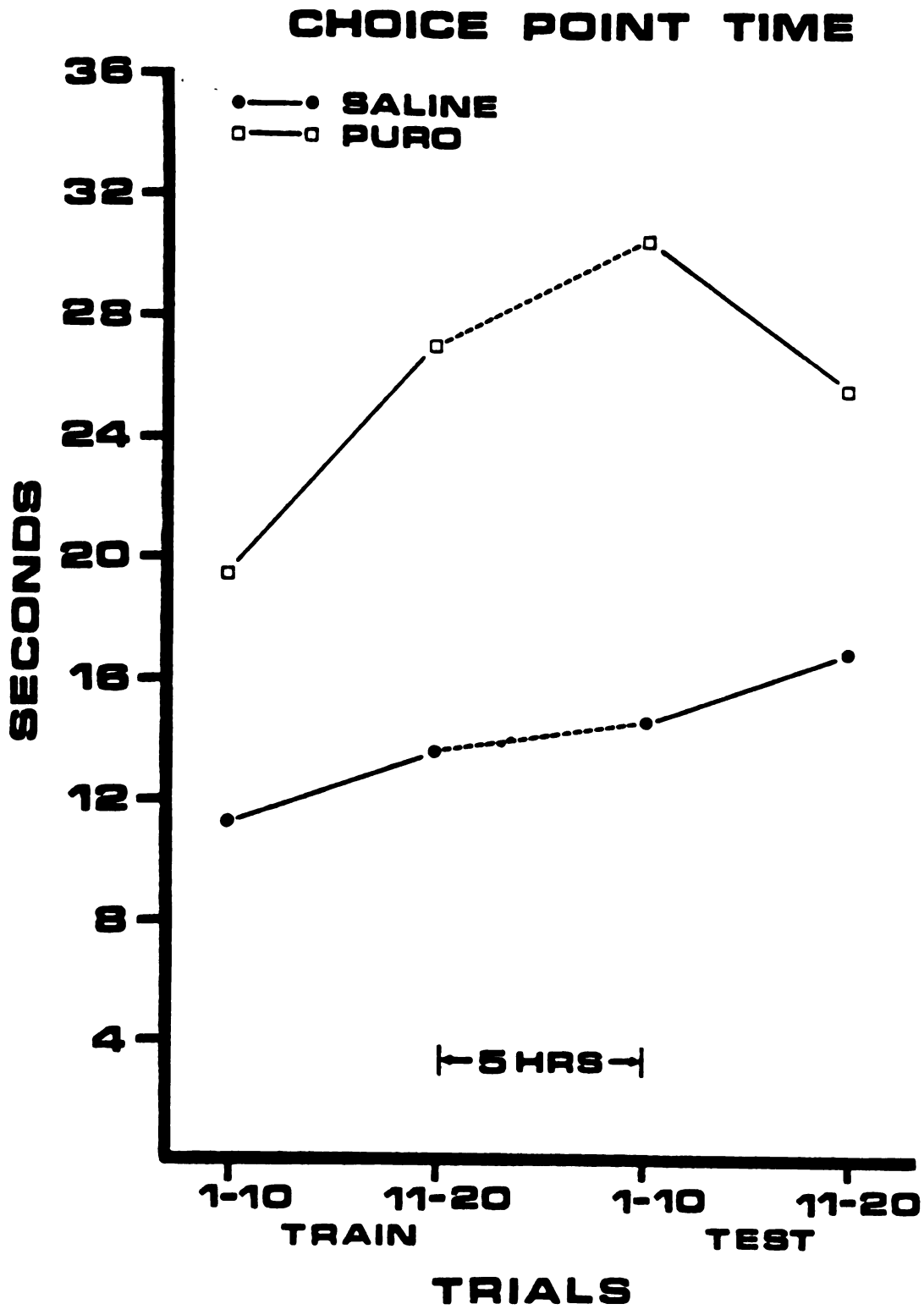
CHOICE POINT TIME
SALINE
PURO
36
32
28
24
20
16
12
8
4
SECONDS
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 9

significant increase in mean choice point time during training; (3) during training trials 11-20 and testing trials 1-10 the PURO group took significantly longer to make its choice than the saline group; (4) the PURO group took a statistically longer time moving from the end of the runway to a choice than in traversing the runway itself, despite the runway distance being approximately one-third of the choice point distance.

The interpretation of choice point time data is less clear than that for runway time. However, several aspects of the results should be considered. One is that the increased choice point time observed for the PURO group when compared to the saline animals cannot represent drug debilitation. This is based on the fact that the PURO animals were as fast or faster than the saline animals in the runway (Figure 8). Similarly, as previously noted, learning was totally unaffected by PURO during training or testing (only retention of the choice behavior from training to testing was affected). Furthermore, the fact that pre-training injections of PURO caused faster runway times but slower choice point times argues against PURO causing its effect on choice behavior (i.e., retention deficits) by increasing the central nervous system activity level of the animal to such an extent that the animal is unable to perform correctly. (Any effect of the drug which might affect general well-being or activity of the animal would be expected to produce changes in the same direction for both runway and choice point times which was not observed.) Thus, it cannot be argued that the amnesia observed for turning behavior produced by pre-training administration of PURO was due either to a general increase in activity level or to general debilitation produced by PURO.

It is also unlikely that the longer choice point times for the PURO group can be explained only in terms of habituation. The reason for this can be seen in Figure 10 in which the "no shock" group mean choice point times (which are presumed to be indicative of habituation) are shown to be essentially identical to the saline animals.

It could be assumed then that the longer choice point times of the PURO group are representative of other factors. One interpretation of this increase is that it is related to the decision-making process, and/or to the process for converting shorter-term memory into longer-term storage. The PURO animals may be forced to "deliberate" longer over an impending choice of the direction to be turned. If this hypothesis is correct it would suggest that pre-training injection of PURO disrupts retention of some memories (while not affecting learning) by interfering with one or more aspects of decision-making and/or longer-term memory consolidation processes.

Post-Training Puromycin (PURO) Administration

Choice Behavior

Post-PURO animals were injected two hours after training (three hours before testing) in the same paradigm as the pre-PURO groups. That is, both groups were trained for 20 trials and then tested for 20 trials five hours after the end of training. A post-PURO group was run to further define the limits of PURO action on memory mechanisms in the cockroach and to draw additional parallels to the vertebrate work in which post-training injections of PURO are known to cause amnesia (see Literature Review).

Figure 10. Mean choice point times during training and testing for animals injected before shock avoidance training with puromycin (PURO) (n=21) or saline (n=12) and a group of animals (n=6) given no injection and exposed to the paradigm for 20 trials of training and testing without any shock applied. The "no shock" animals exhibit statistically identical behavior to the saline group, suggesting that the significantly longer choice point times observed for the PURO animals does not represent habituation.

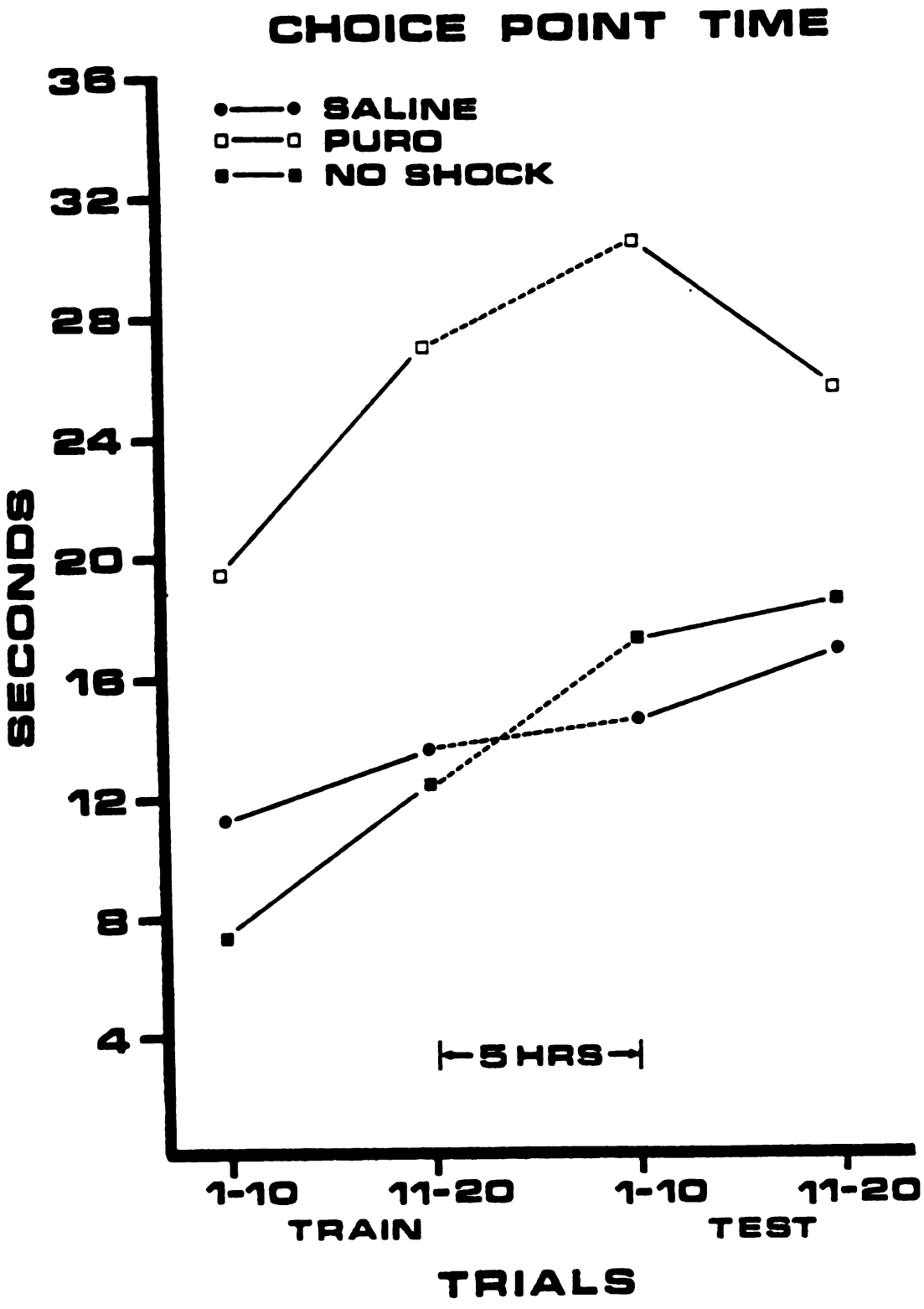
CHOICE POINT TIME
SALINE
PURO
NO SHOCK
SECONDS
36
32
28
24
20
16
12
8
4
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 10

The effect of post-training administration of PURO on choice behavior is contained in Table 1. As evidenced by the statistically significant improvement of 43 percent in the mean number of correct turns between training trials 1-10 and training trials 11-20 the post-PURO animals exhibit excellent acquisition of the shock avoidance training. This was anticipated. The post-PURO group having not received an injection was, in essence, a control group.

However, upon testing (five hours after training and three hours after PURO injection) the post-PURO animals made significantly fewer mean correct turns than the saline animals when statistically comparing testing trials 1-10 of both groups. This retention deficit is also substantiated by the fact that the post-PURO animals do not statistically differ from the amnesic pre-PURO animals in any of the behavioral parameters measured and detailed in Table 1. In addition, the 41 percent loss of retention from training trials 11-20 to testing trials 1-10 for the post-PURO animals is statistically significant ($p < 0.1$, one tail t-test).

The retention deficit produced by post-training injection of PURO is not as apparent when comparing trials to criterion (Table 1). Here the data shows that while the trials to criterion for the post-PURO group is not statistically different from the pre-PURO group (which exhibits amnesia), it is conversely also not statistically different from the saline group (which does not exhibit amnesia). This less dramatic and inconsistent effect of post-PURO treatment in producing a retention deficit can probably be attributed in part to a smaller n value (i.e., 10 post-PURO animals compared to 31 pre-PURO animals) which inherently produces a larger standard error.

It also may be that PURO administered after training is affecting memory mechanisms different from those affected by pre-training PURO injections and therefore might produce a qualitatively different amnesia (see further discussion of pre- and post-PURO effects below).

Runway Time

The results of post-training injection of PURO on mean runway time are contained and compared with other groups in Table 2. Before making comparisons of these times with those of other groups it should again be noted that the post-PURO animals have been injected three hours before testing. As such, this group should behave in a similar manner to the saline group during training and one might possibly expect like the pre-PURO training group during testing since both PURO groups are performing under similar amounts of protein synthesis inhibition at these different times. However, caution should be exercised before drawing any analogies between the runway and choice point times of the pre-PURO training and post-PURO testing groups. The reason being that the post-PURO animals during testing have already been exposed to the maze, undergone substantial learning, and presumably some memory formation. Thus, this may confound any comparisons.

As expected, the post-PURO animals behaved in the runway during training in a similar manner to the saline group. Likewise, as might be predicted, the post-PURO group upon testing exhibited runway times that are statistically indistinguishable from the pre-PURO training times. In addition, the fact that the first ten trials of testing for the post-PURO animals were so much faster than the second ten trials for training seems to confirm that PURO may have an effect on activity,

something that also was observed in the runway times for training trials of the pre-PURO group.

What is somewhat unclear is why the post-PURO animals are the only group of the four represented in Table 2 (10 of 70 animals) that do not show retention of the training runway time habituation. Interestingly enough, all groups, including the post-PURO animals, show additional habituation in runway time during testing. Again, some analogies may be futile due to the difference in the time of drug injection, and possibly mechanisms interfered with. However, it could be that the increase in activity caused by PURO (which was reasoned before to not be responsible for the amnesia of choice behavior) is of sufficient magnitude to counteract the habituation effect of running the maze which slows animals down.

Choice Point Time

The choice point times (Table 2) of the post-PURO are also perplexing in some respects. For instance, while the training choice point times of the post-PURO animals are expectedly similar to the saline animals in showing no increase in time with succeeding trials, they are also unexplainably and statistically longer. Also, the testing choice point times for the post-PURO animals are similar in trend to the times observed for the training trials of the pre-PURO group. However, the choice point times during testing for the post-PURO group are significantly faster than during training, possibly due to the above mentioned activity effect of the drug. It may be that comparisons of the choice point times between the pre- and post-training PURO groups suffer from the same ambiguities mentioned above for comparisons

of the runway times. It also may be that any inconsistencies in these time comparisons between the two PURO groups argues for the different injection times producing retention deficits through different mechanisms (see below).

Summary of Pre- and Post-training Puromycin (PURO) Administration

Including the present study, pre- and post-training injections of PURO has been shown to cause amnesia of at least some longer-term memories in insects, fish, birds and mammals despite differences in neural organization. In addition, very similar maze behaviors have been observed in insects and mammals. For example, the phrase "vicarious trial and error (VTE)," was first used by Meunzinger (1938) to describe the behavior of the white rat at a choice point in a T-maze in which the rat stopped and turned its head back and forth several times before making its turning choice. He related this behavior to the decision-making process. Such behavior may have a behavioral analogue in the cockroach which we also observed to stop at a choice point and turn its antennae and head back and forth and which is related to the relatively long choice point times.

Thus, these experimental results and observed behavioral similarities in the insect and mammal raise the possibility that their underlying learning and memory processes share many common features and further may suggest that the fundamental mechanisms of memory storage evolved several hundred million years ago, before the phyla that these classes represent diverged.

Interpretation of Pre- and Post-training Puromycin (PURO) Results

The first general question that must be raised about the deficits in performance during retention tests produced by pre- and post-training injections of PURO is whether the treatments actually reflect interference with some aspect of memory per se, or whether they can be interpreted as performance deficits due to interference with physiological processes other than memory that are necessary to carry out the responses required. This question resolves itself into two important questions with respect to all drug work on learning and memory. Do the drugs have side effects which significantly influence sensory and/or motor response systems? And if they do, can the experimental results (i.e., retention deficits) be interpreted as resulting from these effects?

As discussed in the Literature Review, PURO in doses usually required to produce amnesia does produce side effects. However, it is not believed that any side effects PURO is capable of producing can account for the memory changes witnesses in this study. The reason being that the animals in this investigation that display retention deficits demonstrate learning and relearning abilities that are as proficient as control animals.

An additional concern is whether environmental factors such as temperature and time of day, which are known to be important variables in behavioral work, could be a component of the amnesia seen. Temperature of the colonies was kept at a fairly constant 24°C and as such could be ruled out as a contributing factor. Similarly, Figure 11 shows the relationship between the average time spent in the runway for all trials of training (for all experimental groups) and the time of day when the

Figure 11. The mean runway times for training trials 1-20 as a function of the time of the day that the training session was initiated and whether animals received puromycin (PURO) or saline before or after shock avoidance training, or no injection and no shock. There does not appear to be a correlation between the time of day or drug injection, and the amount of activity in the maze. Thus, the observed retention deficits exhibited by animals that have been injected with PURO cannot be attributed to effects of the time of day on animal activity.

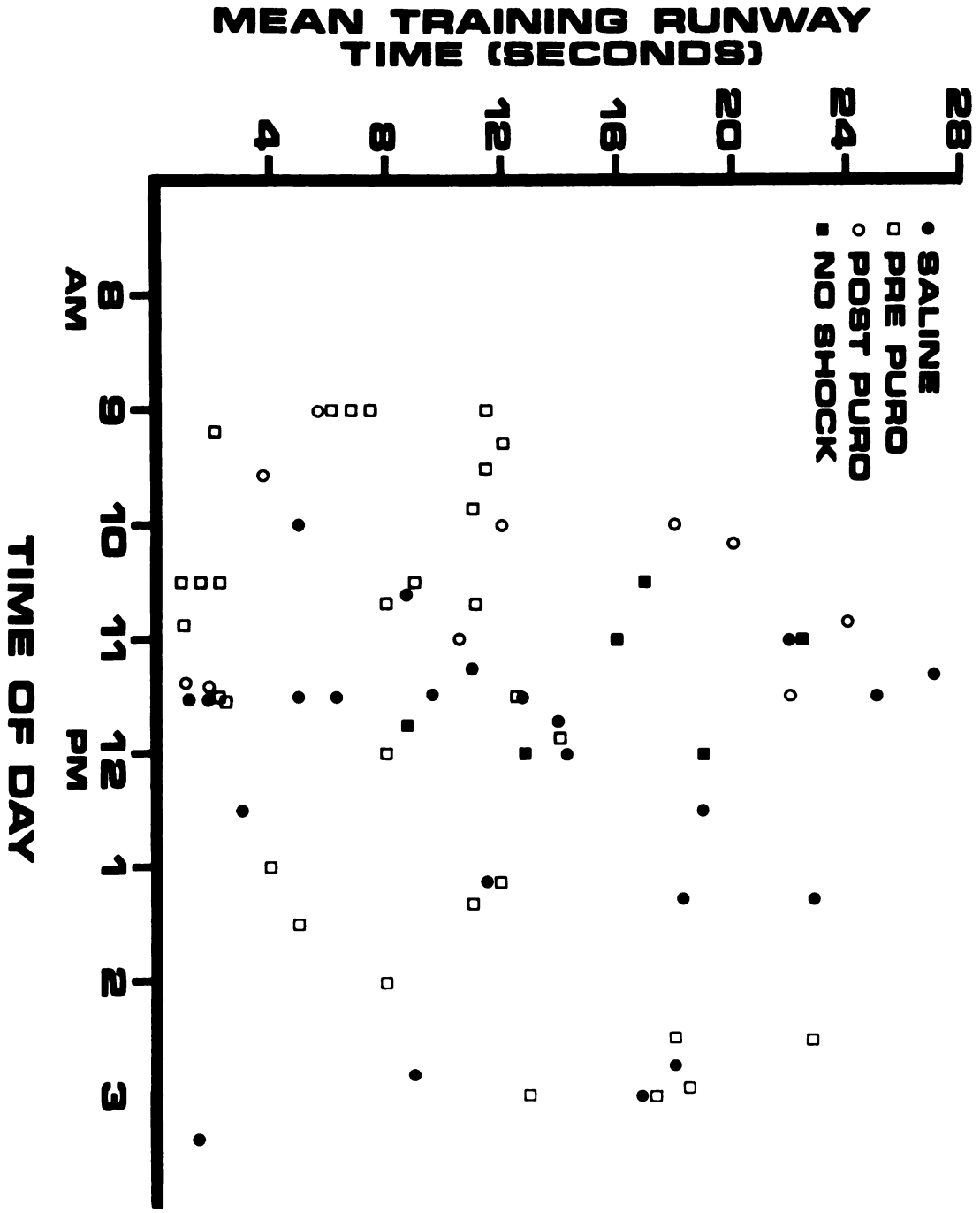
MEAN TRAINING RUNWAY
TIME (SECONDS)
4
8
12
16
20
24
28
SALINE
PRE PURO
POST PURO
NO SHOCK
TIME OF DAY
AM
8
9
10
11
12
PM
1
2
3

Figure 11

training began. A correlation was not discernible. The variable of "time of day" and its effect on "activity" also then can be eliminated as a contributing factor to the amnesia observed. Thus, it would appear that the retention deficits produced by pre- and post-training injections of PURO can best be interpreted as memory deficits.

The next pertinent question that arises from this investigation is what aspect(s) of the memory process is being interfered with by PURO. To what extent does the observed amnesia represent information that only has been registered in the shorter-term form and not consolidated, or represents fully registered and consolidated memory but improperly laid down retrieval processes, or represents blocked retrieval pathways, and so on? The answer(s) to this question has been a major focus of the antibiotic work in the last few years and, frankly, has not resulted in resolution of the issue. Problems that have become apparent in these studies are: (1) it can never be proved that a given memory cannot be recovered; and (2) behavioral criteria can never distinguish whether a given treatment has resulted in a storage or retrieval deficit (Barraco and Stettner, 1976; Miller and Springer, 1973).

However, there have been studies that provide insight into various facet(s) of memory that may allude to processes being affected by PURO. Some of these investigations have attempted to delineate the time course of memory formation. The value of this information is that if it is known approximately how long after training longer-term memory consolidation occurs then it can be inferred that injections of a retention altering drug would most likely be affecting the fixation or retrieval

processes respectively, depending upon whether the drug is administered within or outside of this time span.

Daniels (1971) noted that when AXM was injected five hours before training rats in a one-trail appetive learning task, acquisition was normal and retention was good until about three hours after training. After this time retention gradually decreased until amnesia was considerable at six hours after training. The author concluded that there are at least two memory systems; a shorter-term memory lasting for 0-3 hours, and a longer-term memory lasting 3 hours to 7 days.

Supporting this temporal separation of memory traces is a study by Andry and Luttges (1972). The investigators used a one-trial passive avoidance paradigm and injected mice intraperitoneally with CXM 30 minutes before training or gave electroconvulsive shock (ECS) at various times after training. The mice given ECS 15 seconds after training demonstrated amnesia upon retention testing 1 and 72 hours following ECS. If ECS was administered at intervals greater than 30 minutes it was not able to induce amnesia. The injections of CXM resulted in memory that decayed with time. The retention deficits becoming evident at 2 hours after training and thereafter, 72 hours being the longest interval tested. The conclusion from this study has been that memory formation involves a labile electrochemical phenomenon (shorter-term memory) that over a period of about two hours gradually gives rise to a more stable macromolecular phenomenon (longer-term memory).

As mentioned in the Literature Review, there have been both retrograde amnesia and "Kamin effect" studies in the cockroach that describe memory stages of similar time spans. That is, in the cockroach the conversion from shorter-term to longer-term memory appears

to occur within one to two hours after the cessation of training. Assuming this to be true would imply that the pre-training injections of PURO in this study must be affecting the consolidation of shorter-term to longer-term memory while the injections of PURO two hours after training (post-PURO) are having their greatest effect on the retrieval mechanism.

The assumption that the two injection regimes of this study are affecting essentially different processes, could be tested by further experimentation. It has already been pointed out (see Literature Review) that the differential amnesic effects of PURO in vertebrates can be selectively antagonized. Specifically, simultaneous injections of PURO and amphetamine or PURO and CXM given one or more days after training will not affect retention at later testing trials when injections of PURO alone is known to cause amnesia. However, when PURO is administered simultaneously with either amphetamine or CXM immediately after training, amnesia is apparent at later testing times and its affect on memory is not antagonized. It is these and other data which has led to speculation that PURO is capable of affecting different memory processes such that injections before or soon after training are affecting consolidation processes while delayed injections are perturbing retrieval processes.

While the post-training injections times of this study are not the same as those related above for the vertebrates it may be of value to try to attenuate or antagonize the amnesia produced by pre- and post-training applications of PURO. The experiments involved would be similar to the ones discussed above. That is, one could concomitantly

administer PURO and amphetamine or PURO and CXM three hours before or two hours after training. If it was found that either amphetamine or CXM was able to diminish or counteract the amnesia effect of PURO when injected at one time but not the other then one would be reasonably certain that the different injections were disturbing different aspects of the invertebrate memory consolidation-retrieval process.

Possible Mechanisms of Puromycin (PURO) Action

While it is known that PURO can act as an amnesic agent in both vertebrates and invertebrates by affecting consolidation and/or retrieval processes, this information has provided little resolvement as to the molecular basis of memory. The reason for this is that as yet no one has been able to precisely define the mechanism(s) by which PURO is causing the retention deficits. As previously stated, the original and guiding premise of the antibiotic work has been that antibiotics impair memory by inhibition of protein synthesis, thereby preventing the establishment of memory traces necessary for longer-term memory storage. However, the research presented here and implied elsewhere has discouraged such simplistic conclusions. Indeed, for the last few years researchers have investigated other actions of PURO that may account for the retention deficits observed.

One of these areas of research has been the possible interaction of PURO with neurotransmitter systems. It was presented in the Literature Review that neurotransmitters are known to be in some way involved in both learning and memory. Also, work has been cited above that hints at an interaction between the adrenergic systems and post-training injections of PURO. In addition, there are other investigations that

allude to a relationship between the metabolism of putative neurotransmitters and actions of PURO. For instance, PURO is known to depress cholinesterase synthesis (Burkhalter, 1963). There is also evidence that PURO is an effective inhibiter of rat brain cholinesterase and that it selectively binds at two classes of sites on the enzyme, one of which may be an allosteric site. Since the allosteric site of cholinesterase has similar properties to the acetylcholine receptor and appears to bind to most ligands that bind to the receptor, this evidence supports the idea that PURO also may block the effect of acetylcholine at the synaptic membrane (Moss, et al., 1974). This theory is further corroborated by work that found PURO blocked the post-junctional response of acetylcholine in a frog muscle preparation (Wulff, 1973).

Peptidyl-PURO, the abnormal peptides containing PURO that are released from the protein synthetic machinery, also have been implicated in producing amnesia through adrenergic neurotransmitter interference. Roberts, et al., (1970) believe that the possibility of this involvement is suggested by the similarity of the chemical structure of the O-methyltyresine moiety of PURO to neropinephrine and various mescalin-like drugs. The authors feel that post-training PURO-induced amnesia may be the result of peptidyl-PURO fragments that bind to norepinephrine receptor sites at synapses and block the expression of memory. (The inference in this case is that some stable modification of adrenergic sites are involved in the formation and persistence of the memory trace.) Hence, the authors believe that this could account for the injections of saline restoring memory by "washing out" the peptidyl-PURO fragments,

or in the case of drugs which stimulate the adrenergic system and attenuate the amnesia by overwhelming the peptidyl-PURO blockage.

There have been enough apparent correlations between the effects of PURO and neurotransmitter systems that Barraco and Stettner (1976) have proposed a tentative model to describe antibiotic-induced retention deficits which does not necessarily include protein synthesis inhibiting effects of antibiotics as causing these deficits. Rather, the model emphasizes important interactions of PURO (as well as CXM) with neurotransmission. In this model, the cholinergic system represents information storage (memory), while the adrenergic system represents information retrieval integration. Further, the adrenergic system exerts an "activational" effect on the neuronal networks that are associated with the acquisition process during training, in addition to causing preferential activation of these networks during testing (e.g., retrieval process). The adrenergic changes could lead to permanent alterations of the cholinergic networks, thus establishing the memory trace. This suggested involvement of both transmitter systems in learning and memory processes is not inconsistent if it is considered that the adrenergic systems act as modulator to increase the sensitivity of the cholinergic system (Roberts, et al., 1970), or that acetylcholine is involved in the synaptic mechanisms responsible for the release of norepinephrine (Burn, 1968). Additionally, there has been a model for learning outlined in which a biogenic amine, released generally throughout the brain during certain affective states, that facilitates the increase efficiency of synapses recently activated (Kety, 1970).

According to Barraco and Stettner, this neurochemical system interacts with a "continuum of preparedness," which is 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 easily learn) to associate a defensive action, such as escape with shock avoidance, while they are unprepared (and have a difficulty learning) to associate an apetitive response with shock avoidance. Thus, according to the model, the adrenergic activational effect would participate less in the learning of highly "prepared" tasks, in which the stimulus-response associations are highly "primed" within the species-specific repertory of behavior, and conversely, participate more in the unprepared and contraprepared tasks in which the stimulus-response associations are more "phylogenetically" novel. Superimposed upon this preparedness continuum would be the level of training; overtraining produces, in effect, highly prepared associations 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. The adrenergic effects of antibiotics (e.g., CXM and delayed PURO injections) would thus essentially vary quantitatively, and since they involve retrieval systems and never totally block adrenergic activity, their amnesic effect would always be potentially reversible. The cholinergic effects of antibiotics (e.g., PURO before or immediately after training) would,

however, produce interference with memory storage resulting in permanent amnesia.

Pre- and Post-Training Scopolamine Administration

Scopolamine is known to block muscarinic receptors in the brains of vertebrates (Goodman and Gilman, 1975). It is also known to produce retention deficits (see Literature Review for details). As a result, the cholinergic system has been implicated in the memory process. In addition, as mentioned above, there has been a model of memory disruption by antibiotics that postulates pertubation of the cholinergic system as being a possible cause of the retention deficits produced by injections of PURO before or immediately after training. If the pre- and/or post-training injections of PURO in this study produce amnesia by interfering with the cholinergic system then it follows that injections of agents that block the cholinergic system should mimic the results of the PURO injection(s). Such a finding would at least indicate a possible correlation between the action of PURO and cholinergic transmission. The relationship could then be strengthened by future experimentation. Thus, in an attempt to determine what influence cholinergic transmission has on learning and memory processes in the cockroach, and to assess if the results of this study in which PURO disrupted cockroach memory could be explained in terms of PURO effects on cholinergic transmission, individual groups of animals were injected with scopolamine either one hour before training or one hour before testing (four hours after training) in the aforedescribed paradigm.

The effects of the injection of scopolamine are contained in Figure 12 and Table 3. The results show that muscarinic blocking

Figure 12. Mean number of correct turns during training and testing for animals injected with scopolamine one hour before shock avoidance training (n=19), scopolamine one hour before testing (n=6), or saline one hour before training or one hour before testing (n=13). The curves are not significantly different from one another at any point graphed. Standard errors are given in Table 3.

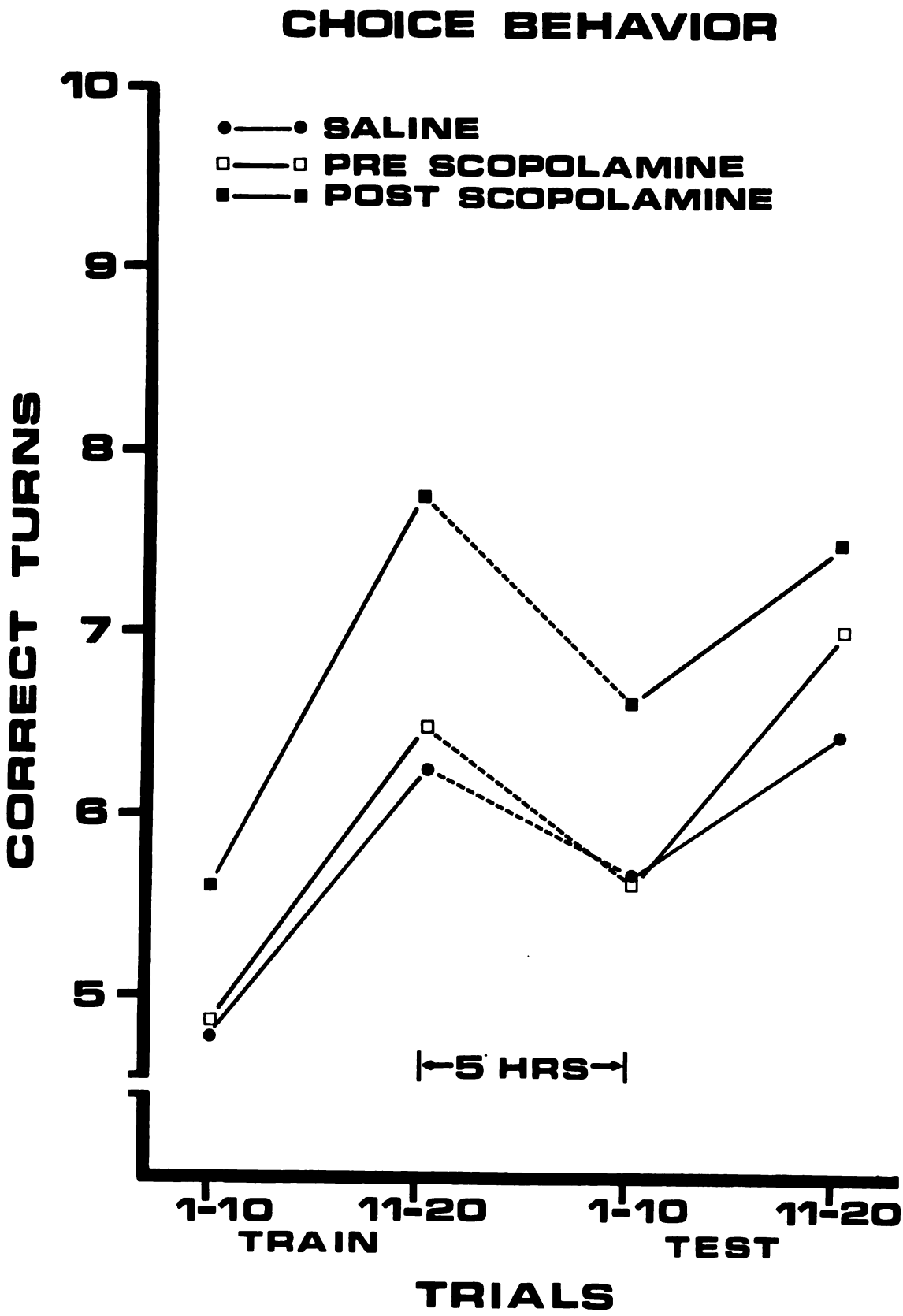
CHOICE BEHAVIOR
SALINE
PRE SCOPOLAMINE
POST SCOPOLAMINE
CORRECT TURNS
10
9
8
7
6
5
5 HRS
1-10
11-20
1-10
11-20
TRAIN
TEST
TRIALS

Figure 12

Table 3. Comparison of mean number of correct turns and trials to criterion during training and testing for animals injected with scopolamine or saline one hour before or one hour after shock avoidance training. All animals were given 20 training trials followed five hours later by 20 testing trials.

	TRAINING				TESTING			
Treatment (n)	Correct Turns 1-10	Correct Turns 11-20	%improvement	Trials to 5/6	Correct Turns 1-10	Correct Turns 11-20	%loss	Trials to 5/6
Saline (13)	3.85 ± .45[a]	6.23 ± .62	62[b]	15.31 ± 1.65[c]	5.75 ± .62	6.33 ± .54	20[d]	13.25 ± 1.86
Pre-Scopolamine (19)	4.23 ± .40	6.53 ± .40	51	15.47 ± .98	5.68 ± .45	7.00 ± .46	38	11.68 ± 1.14
Post-Scopolamine (6)	5.67 ± .56	7.83 ± .70	38	13.33 ± 2.22	6.67 ± .49	7.50 ± .34	54	10.67 ± 1.89

[a] Mean ± S.E.M.

[b] % improvement is calculated by subtracting the mean correct turns for training trials 1-10 from mean correct turns for training trials 11-20 and dividing by the mean correct turns of training trials 1-10

[c] refers to the number of trials that the animal took to reach a criterion of 5 out of 6 correct turns

[d] % loss is calculated by subtracting the mean correct turns for testing trials 1-10 from the mean correct turns for training trials 11-20 and dividing by the difference in the mean number of co-rect turns of training trials 11-20 and training trials 1-10.

on cholinergic receptors before and after shock avoidance training did not significantly effect acquisition or retention of the paradigm when comparing absolute numbers of the mean correct turns and mean trials to criterion for these groups with a saline-control group (separate from the one run with PURO). However, when comparing the percent improvement and percent loss of correct turns during training and testing trials the saline animals appear to demonstrate somewhat better acquisition and retention of the training (Table 3). In spite of this apparent contradiction, the slopes of the training and testing curves for both groups appear remarkably similar. Thus, the present, tentative conclusion is that scopolamine administered both before and after training does not affect learning or memory of this paradigm in the cockroach. The previously mentioned work with PURO, utilizing the same paradigm, indicated that retention but not learning was affected by pre-training injections of PURO and that retention was affected by post-training administration of PURO. Taken together, the PURO and scopolamine results suggest that PURO may not be interfering with cholinergic synapses in the central nervous system of the cockroach to produce its amnesic effects.

However, the scopolamine results must, at present, be viewed as preliminary. The reasons for assigning this status to the work are:

1. low initial learning values for the pre-training scopolamine and saline groups, even though the percent improvement between the first and second ten trials of training were comparable to previous groups run. This may in part be due to the fact that at the time of the pre-training scopolamine and most of the saline experiments our lab was in an animal shortage crisis and thus had to rely on

several different sources to supply the cockroaches, some of whose quality was suspect. This feeling is supported by the results of the post-training scopolamine group. These animals exhibited training behavior that closely resembled previous groups, and were obtained from our eventually thriving homogeneous colony.

2. the group of animals injected with scopolamine after training represents a "n" of only six animals which may be an insufficient number to judge the effect of any drug.
3. lack of proof that scopolamine in other possibly higher doses was incapable of interfering with memory processes. Before stating categorically that a drug is not having an anticipated effect it is necessary to run a dose-response curve. Our toxicity study did indicate that the dose of scopolamine used was, according to the criteria outlined, the largest that could be administered. In addition, evidence that the drug was acting centrally was indicated by faster runway times for both of the scopolamine groups, as well as the occasional observance of motor disturbances of some animals (i.e., from a loss of coordination to the appearance of initial stages of anesthesia). Since there are no known perpherial cholinergic synapses in the cockroach these effects had to be of central origin. However, thought should be given to administering larger doses of scopolamine (since there was a rather large therapeutic index) in an effort to find out if learning and memory can be affected by any dose. If effects on learning and/or memory are discovered then experiments could be designed to distinguish whether the observed effects were due to actual

pertubation of learning and memory processes or simply the result of unrelated secondary effects of the drug.

In sum, further experimentation elucidating any involvement of either the cholinergic or adrenergic systems in learning and memory processes in the cockroach would be welcomed. These could include both attempts at attenuating or enhancing neurotransmission. Successful cholinergic blockers that have been used in the vertebrates include scopolamine and atropine, with scopolamine thought to be the more effective central blocker (Goodman and Gilman, 1976). A cholinergic enhancer that has proved useful in vertebrate studies is the cholinesterase inhibitor physostigmine. A potent adrenergic blockage can be accomplished by a combination of alpha-methyl-p-tyrosine and reserpine, while amphetamines appear to be the more accepted adrenergic potentiator.

Attempts should also be made to relate any possible effects of these agents with the action of PURO. For instance, if a relation between blocking cholinergic synapses and either injection regime of PURO (pre- and/or post-training) is indicated, then enhancers of cholinergic transmission such as physostigmine might be capable of antagonizing the PURO effect. Amphetamine could be similarly used (and already has been in vertebrates) to further disclose an adrenergic-PURO relationship.

The use of antibiotics such as PURO to explore the molecular basis of memory is at a critical point. Such experiments, which originally held so much promise, but are now frustrating researchers, must not be abandoned. Puromycin is undeniably capable of experimentally producing behavioral amnesia. Thus, it is potentially a powerful tool in the

elucidation of memory processes. What is needed is a refocusing of efforts away from simplistic biochemical interpretation of the drug's effect (e.g., protein synthesis inhibition) to an intensified exploration of the drug's known and unknown secondary effects. As stated, this could initially involve research into areas such as the interaction of PURO and neurotransmission. For when we arrive at the actual mechanism by which PURO does produce amnesia we will have acquired the first key to the first door to understanding the chemistry of memory formation and ultimately the nature of thought itself.

CONCLUSIONS

1. Puromycin injected before training in a shock avoidance paradigm does not affect acquisition but does produce retention deficits of the correct choice behavior when the animal is tested five hours after the end of training.
2. Puromycin injected before training does not affect acquisition or retention of the habituation that is reflected by a progressive increase in the runway time.
3. It would thus appear that PURO may be specific for the different types of longer-term memories that may accompany any shock avoidance training situation.
4. It is speculated that the types of behavioral modification represented by learning of the correct choice behavior, retention of the correct choice behavior, and increased runway time probably are mediated at different levels within the central nervous system and very likely involve different mechanisms that differ in their susceptability to PURO action.
5. It is possible that the increase in choice point time taken by the animals injected with puromycin before training is related to the decision-making process and thus represents another form of behavioral modification that is measurable in this experimental paradigm and may reflect the action of PURO. Its interpretation at this time remains unclear.
6. Puromycin injected two hours after training (three hours before testing) also produces amnesia of the correct choice behavior upon testing.

7. It is postulated that the retention deficits produced by the pre-training injections of PURO reflect interference of the memory consolidation process, while the post-training injections are felt to be having their greatest effect on the retrieval mechanism.
8. The exact process by which PURO is causing the retention deficits is at this point unknown. However, total protein synthesis inhibition, debilitation of the animals due to sickness, and central nervous system activity effects can be ruled out as causitive factors. In addition, preliminary experiments utilizing scopolamine injections and the same paradigm suggests that the amnesia observed with pre- and post-training administration of PURO is unlikely to be the result of interference of central cholinergic synapses.

This work, to date, strongly supports similarities between the learning and memory behaviors of the invertebrate and vertebrate. It suggests the possibility that the underlying mechanisms from the insect to the mammal probably have many common mechanisms and may further indicate that the fundamental mechanisms of learning and associative memory storage must have evolved several hundred million years ago when the phyla that these two classes represent diverged.

APPENDIX A

APPENDIX A

Folin-Lowry Protein Assay

Solution A = 20g Sodium carbonate in 1L of 0.1N NaOH

Solution B = 10g $CuSO \cdot 5H_2O$ made up to 1L with distilled water

Solution C = 20g sodium tartrate made up to 1L with distilled water

Folin Phenol reagent. (Store in refrigerator)

Solution D = 0.5ml B + 0.5ml C + 50ml A in that order!

Solution E = Folin Phenol diluted 1:1 with water before use

Procedure

1. Set up sample tubes bringing each sample volume up to 1.0ml with 0.1N NaOH. In the case of the sample from the cockroach nervous system homongenate (0.5ml) add 0.5ml.

2. Add 5.0ml of reagent D, mix and incubate 30 min in a 45° water bath.

3. Add 0.5ml or reagent E, immediately mix vigorously (reagent decomposes) and let stand at room temperature for 30 min.

4. Read on Beckman spectrophotometer at 620nm.

Compare the results with the standard curve run with known quantities of protein from BSA (Bovine Serum Albumin) samples to determine amount of protein in sample.

Folin-Lowry Standard Curves Points Using BSA

mgy-BSA	Absorbance @ 620 nm
0.10	0.30
0.20	0.52
0.30	0.71
0.40	0.90
0.60	1.20

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