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DISCLAIMER

PROTEIN SYNTHESIS AND MEMORY STUDIED WITH ANISOMYCIN

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SUMMARY

This paper considers a number of aspects of research using inhibitors of protein synthesis to determine the role of such synthesis in formation of long-term memory. The importance of aspects of the behavioral tasks is discussed, and the relative value of anisomycin and other inhibitors is considered. Several alternatives or arguments against the protein-synthesis hypothesis are rebutted. Our review indicates that single experiments prove little; parametric research varying systematically such factors as strength of training and magnitude of inhibition, as well as use of appropriate control experiments, is necessary.

INTRODUCTION

At a symposium on Protein Synthesis in the Brain, there is perhaps no more important and challenging topic than the role of cerebral protein synthesis for memory. Even after more than a quarter of a century of speculation and research on the possible involvement of protein in memory trace formation, far more questions remain unanswered.[1-5]

The use of inhibitors of a biochemical reaction to study the role and necessity of that reaction is a technique that is common and accepted; so it was to be expected that researchers would turn to inhibitors of protein synthesis as one strategy to study the role of such synthesis in memory. In concept, such experiments are simple--train an animal on a behavioral task, administer an inhibitor close to the time of training, and later test the animal for its retention (or memory) of the prior training. In practice the problem is more difficult, as many limitations and complexities exist in selection and execution of the behavioral task, in the selection of drug, and finally in the interpretation of experiments.

BEHAVIORAL TASK

To test the effect of a prior experience on behavior (that is, to test for memory) it is essential to have a suitable behavioral test, or preferably several tests to check on generality of results. The details of behavioral tests will vary with the organism. Conceptually, most commonly used behavioral tests are rather simple, although in practice they can become complicated and sophisticated.

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Fig. 1. Schematic representation of three
types of behavioral apparatuses useful for
studying memory. The step-thru test can
be used as either a passive[19,23] or an
active[4] avoidance test. In the passive
avoidance test, the animal must remain in
the small compartment on the left to avoid
shock; in the active avoidance test, the
animal must move to the large compartment
on the right, frequently only after a cue
such as a bell or light, to avoid shock.
For the pole jump[33], the animal must jump
onto the pole to avoid shock. The T-maze
may be used as either a spatial (right-
left)[22] or a visual (light-dark) discrim-
ination task.

POLE-JUMP

STEP-THRU

T-MAZE
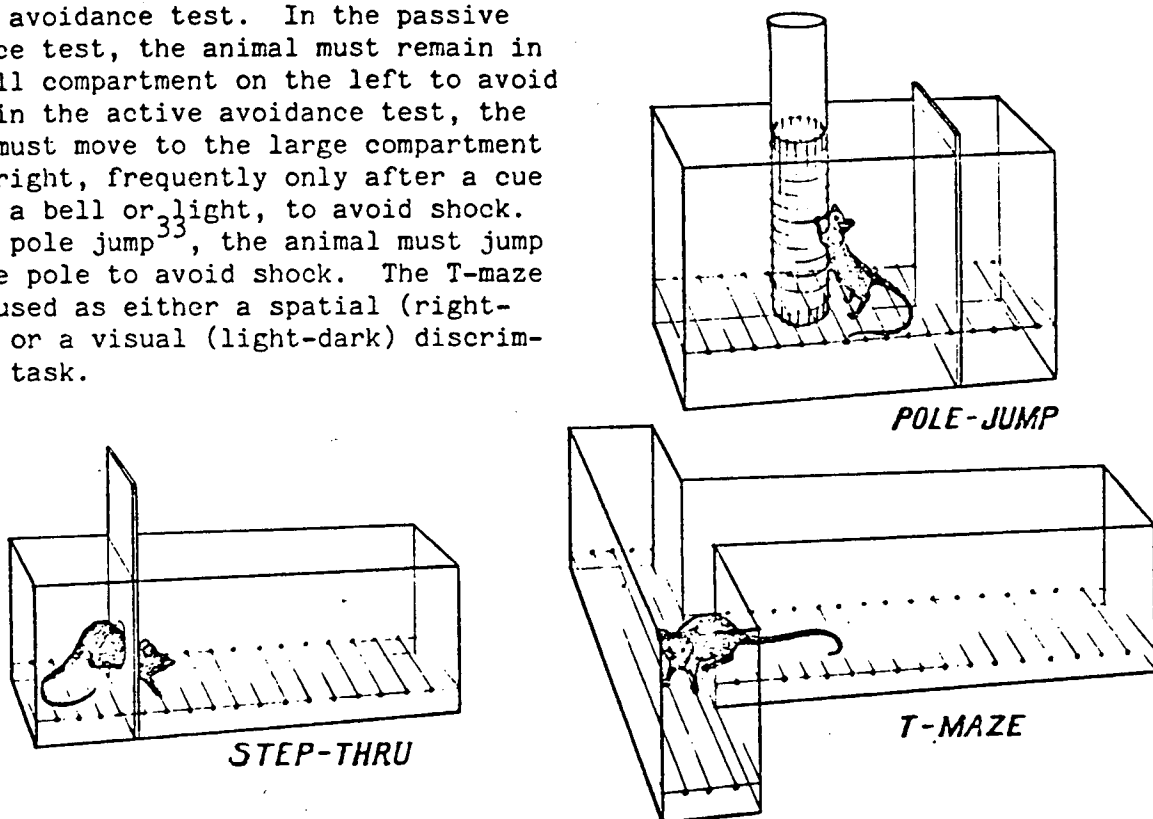
XBL 763-5751

A parallel can be found in many common neurochemical procedures which can be
stated simply but where actual techniques are critical. Fig. 1 presents schema-
tic drawings of 3 test apparatuses commonly used with laboratory rodents. The
training procedures for these tasks vary in difficulty; for the step-through pas-
sive avoidance or active avoidance tasks, a high level of retention can typically
be demonstrated after only one trial. That is, in the case of step-through pas-
sive avoidance, the animal needs only one trial to learn and remember not to step
into the shock compartment. The pole-jump task may require 3-5 trials before an
animal will learn to jump onto the pole at the sound of the warning buzzer to
avoid or escape shock. A T-maze procedure may require 15-20 trials before an
animal will learn to escape shock by running to the correct arm after the cue.
Many factors, some of which are subtle, can influence training and testing of the
subjects and consequently the reproducibility of experiments. These include not
only such factors as strength and duration of shock, but also details of handling
the animals. Strain differences within a species is a major variable. Rigorous
control of these factors must be exercised in order to get reproducible results.[4]

Fig. 2. Anisomycin (ANI)
2-p-methoxyphenylmethyl-3-acetoxy-4-
hydroxypyrollidine. $C_{14}H_{19}NO_4$
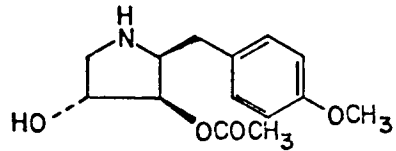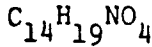
INHIBITORS OF PROTEIN SYNTHESIS

After test subjects and suitable tasks have been selected, there remains the
problem of selection of an appropriate inhibitor of protein synthesis (PS). The
list of inhibitors is steadily growing, and there are now more than 100, only
half of which are effective in eukaryotes.[6] The list of useful inhibitors for
psychobiological studies is further reduced by many additional constraints includ-
ing availability, solubility, toxicity, side effects, etc. At present, less than
10 inhibitors of PS have been used in studies of memory and most research has
been done with four--puromycin (PURO), cycloheximide (CYCLO), acetoxycycloheximide
(AXM), and recently anisomycin (ANI) (Fig. 2). Two other inhibitors, pactamycin
and emetine, have been used to demonstrate the necessity of cerebral as opposed
to peripheral inhibition.[7,8] The modes of action of each of the main inhibitors
are established[6].

Puromycin was the first PS inhibitor used in behavioral experiments. In now
classic experiments, Flexner et al. reported that cerebral administration of PURO
which produced long-lasting inhibition of PS also impaired memory even when
administered hours or days after training.[9,10] Studies with PURO and the glutari-
mides (CYCLO and AXM) were extended to the goldfish by Agranoff et al.[11,12]
About the same time, the glutarimides were used in mice, primarily by Barondes
et al.[1,13] While many important experiments had been done with PURO, AXM, and
CYCLO, each of these inhibitors had drawbacks for many behavioral studies in mice
and other rodents: PURO must be administered intracerebrally which is a compli-
cating factor for many behavioral experiments, and PURO produces abnormal
electrophysiological activity[14,15] which complicates the interpretation and signi-
ficance of the result. AXM and CYCLO share the advantage of being effective even
when administered peripherally, but unfortunately AXM is no longer available, and
CYCLO must be administered at near-toxic doses to mice in order to produce both
a high degree of PS inhibition and amnesia. In addition, high toxicity greatly
limits the design of experiments employing CYCLO.[4,16]

Although many investigators (including ourselves) believe, based on research
with inhibitors, that PS is required for formation of long-term memory, critics
remained unconvinced and raised counterarguments such as the following: 1) Inhi-
bitors are not effective in well-trained subjects.[5] 2) The amnesic effects of
inhibitors of PS can be most readily accounted for in terms of an impairment of
retrieval mechanisms.[2,5] 3) The critical deficiency is in a protein required
for normal neural function, rather than for consolidation of memory.[5] 4)Altera-

tions in catecholamine turnover produced by PS inhibition may be a major cause of amnesia.[5,17] 5) Peripheral PS inhibition prevents an essential elevation of corticosterone during training and it is this lack of corticosterone elevation that is the cause of amnesia.[18] 6) Generality of effects cannot be shown across species or even among species as closely related as mice and rats. These arguments will be rebutted later in this paper.

In order to find an inhibitor that would circumvent some of the disadvantages of those previously used, we initiated in 1968 a search for other inhibitors of PS useful for studies of memory formation. Pactamycin and emetine were found to be poor inhibitors in the CNS, streptovitacin A (a glutarimide derivative), was effective only if administered intracerebrally, but under these conditions was a potent PS inhibitor and a highly effective amnestic agent in rats.[7] In contrast, anisomycin (ANI) was found to have highly desirable properties for use in mice[4] and chicks.[16] ANI is a potent inhibitor of PS through its interference with transpeptidation.[6] As little as 0.5 mg will inhibit cerebral PS more than 80% for 2 h and the onset of inhibition is rapid. It is relatively non-toxic in mice, perhaps because inhibition is less in liver than in brain.[19] The LD_{50} for subcutaneous administration is greater than 20 mg/mouse. Repeated doses of ANI can be administered to maintain a high level of PS inhibition for extended periods and, rather surprisingly, inhibition of cerebral PS for as long as 12 to 14 h is not lethal to mice, and the observed side effects are minimal. These properties of ANI have made it a particularly valuable tool to investigate many questions concerning long-term memory formation,[4,8,16,20,21] and many investigations of the amnesic effects of ANI have now been reported. In the following sections, we will review some experiments using ANI which address alternative explanations that have been raised concerning the role of PS in memory.

ANSWERS TO ARGUMENTS AGAINST THE PROTEIN SYNTHESIS HYPOTHESIS

1) <u>Can amnesia be demonstrated in well-trained subjects?</u> Using ANI, the duration of inhibition can be controlled and extended by administering doses every 2 h, so it has been possible to investigate whether more prolonged inhibition of PS would produce amnesia in better trained animals. With a given behavioral task, many factors, including the duration and intensity of the training shock and the number of training trials, markedly influenced the degree of training or the "training strength." With a given duration of PS inhibition, amnesia at testing was demonstrated to be inversely related to training strength. For a given training strength, amnesia was positively related to the duration of PS inhibition.[4,19] Experiments employing the step-through avoidance task demonstrated that under conditions where control of parameters yields strong training, as much as 6 h of PS inhibition was required to obtain a moderate degree of amnesia;on the other hand, when training strength was kept low, only 2 h of inhibition was required to pro-

duce amnesia. In other experiments, using learning tasks requiring more training (e.g. T-maze) as much as 14 h of inhibition was required to demonstrate amnesia.[22] We now believe that, within practical limits of duration and degree of inhibition of brain protein synthesis, it is possible to demonstrate that for any increase in training strength that blocks amnesia, a duration of inhibition exists that will reestablish the amnesia. In other words, even well-trained subjects will become amnesic if the period of inhibition is prolonged sufficiently.

2) <u>Does inhibition of protein synthesis impair retrieval processes rather than impairing memory trace formation?</u> Some evidence has been offered that even when PS inhibition has followed training and amnesia has resulted, the memory may later be revived, either spontaneously or by means of special reminder techniques; in that case, the earlier amnesia would not show that a long-term memory trace did not exist but only that retrieval had not been effective.[2,5] In weighing the alternative interpretations, we believe it is important to realize that strengths of different memories vary, some being close.to the threshold for a given behavioral criterion, some being above and some being below (Fig. 3). When it is considered that an amnestic agent can have a graded effect upon memory as a function of numerous variables (e.g., drug-dosage, training strength, interval between training and testing, etc) it is not surprising to find cases of recovery when a amnesia is only partial and memory strength is only slightly below threshold. It should be noted that spontaneous recovery has only been obtained from amnesia occurring at 24 h after training. No spontaneous recovery from amnesia tested 1 week or longer after training has been observed. The less effective the amnesic treatment, the more susceptible it is to reversal; thus recovery is more readily demonstrated from amnesia produced by catecholamine inhibition than by PS inhibition. No evidence of spontaneous recovery has been observed after ECS treatment which had been given shortly after training. In general, amnesias are easier to reverse after the animal has received strong training and a minimum disruptive treatment. According to the model presented in Fig. 3, memory traces
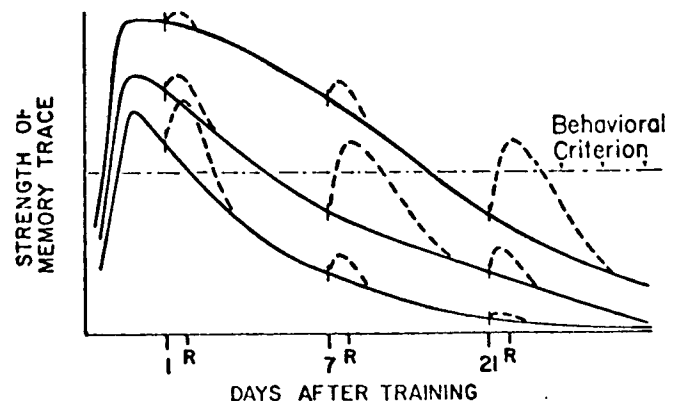
MEMORY STRENGTH

Fig. 3. Schematic representation of the strengths of memory traces as a function of time after training and the initial training strength. Appropriate treatments may strengthen poorly formed memory traces and raise the strength above the threshold level of the behavorial criterion.

weaken as a function of time. A partial or weak memory can be pushed above
threshold by a drug at the time of testing or by use of repeated testing. But we
have found that if the memory is very weak, either because of lapse of time or be-
cause of inhibition of PS, then even reminder treatments do not produce recall.[23]
Thus the model accounts for both cases where reminder treatments are effective
and for the cases where they are not. Not only can the memory consolidation hypo-
thesis explain recovery from amnesia, but it also accounts for the time-dependent
nature of memory formation, whereas the retrieval hypothesis ignores this. We
believe, therefore, that the consolidation hypothesis offers the most parsimon-
ious explanation for memory trace formation, interference by amnestic agents, and
recovery from amnesia.

3) Does PS inhibition interfere with proteins essential for normal brain func-
tioning rather than affecting consolidation of long-term memory? It has been
argued that inhibitors of protein synthesis may alter the amount of some protein
essential for normal brain function.[5] For this argument to be tenable, it is
necessary to show that the time course of these postulated alterations matches the
time course derived from behavioral experiments using inhibitors of protein
synthesis. No systematic work of this type has been published to support this
hypothesis. Among many types of evidence that can be marshalled against this
poorly supported hypothesis, we will summarize only one here and will refer to
others.

We have measured the minimum duration of protein synthesis required immediately
after training to block the amnesic effect of ANI.[24,25] Groups of mice were
administered ANI at 15, 5, or 3 min or 30 sec prior to training on the one-trial
active avoidance task. Strong training was given, employing a relatively high
shock intensity. Biochemical experiments showed that greater than 80% PS inhibi-
tion was achieved within 2-3 min after administration of ANI. After training, all
animals received 3 additional injections of ANI at 2 h intervals to extend the in-
hibition for 8 h. Under these conditions, a high percentage of amnesia was
observed in all groups except for the group administered ANI 30 sec prior to train-
ing (Fig. 4). In this group, PS proceeded for a few minutes after training before
the inhibitor took full effect, and amnesia was prevented. Barondes[11] had put an
upper limit of about 8 min required for PS after training to prevent amnesia;
his experiment, however, used multiple trial training in which time of training
could not be as closely defined as in single-trial training. We believe that it
is very unlikely that the concentration of a protein essential for normal brain
function such as those for neurotransmitter metabolism would change markedly with-
in a very few minutes after administration of a PS inhibitor. Also, we are un-
aware of any enzymes that ANI strongly inhibits except those involved in protein
synthesis. In addition, if depletion of enzymes or other constitutive brain
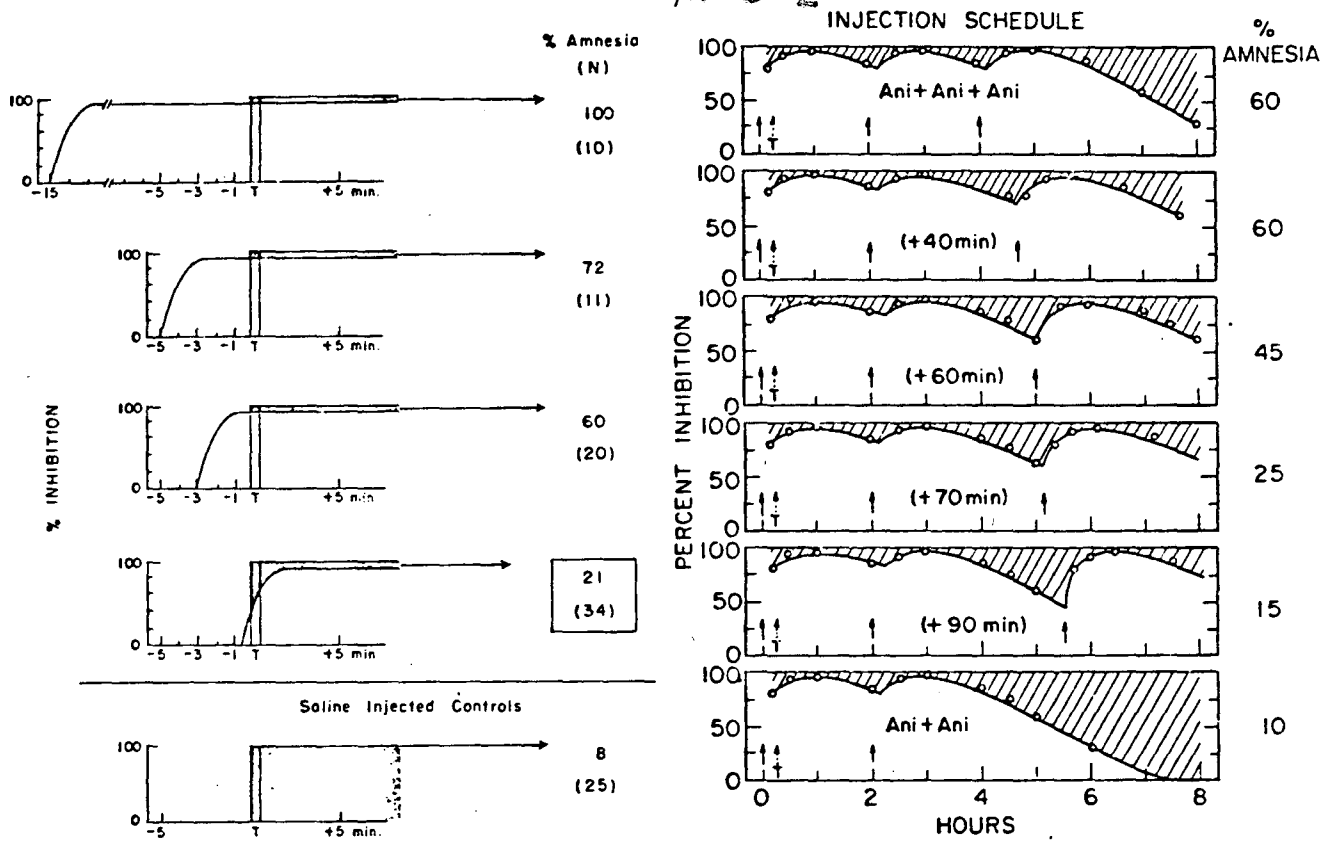
Figure 4. (left) Time course of inhibition of protein synthesis by ANI admini-
stered at several times prior to training and the resulting percentage of amnesic
mice (shown in the right hand column). Under conditions of strong training, only
the control group and the group administered ANI 30 sec prior to training failed
to show amnesia. Number of mice per group is given in parentheses. It is con-
cluded that a brief period of PS shortly after training can suffice to prevent
amnesia.

Fig. 5. (right) Effect on amnesia of increasing the duration of partial inhibition
of PS (indicated by the shaded area) by delaying a third injection of ANI (begin-
ning 4 h after training). As the interval between the second and third injection is
lengthened, the percentage of amnesic mice decreased. This demonstrates that under
these conditions, the potential for establishing long-term memory may exist for
4-6 h after training.

proteins at the time of learning were the mode of action of PS inhibitors, then

prolonged inhibition prior to learning should be equally or more effective than

post-training inhibition in producing amnesia. But studies have shown that when

the training task is performed at the end of a lengthy period of PS inhibition, no

evidence of amnesia was obtained.[5,26]

If any investigator wishes to work out the time course of effect of inhibitors

on enzymes or neurotransmitters, they will be able to see if their results fit

the highly specific curves available not only from the experiments mentioned

above but also from experiments in which amnesia has been shown to be related to

short periods of partial protein synthesis placed at particular intervals after

training (Fig. 5), as well as the many experiments demonstrating that amnesia is

a joint function of training strength and duration of inhibition.

4) *Is alteration of catecholamine metabolism the critical effect of PS inhibitors?* A related hypothesis is that changes in catecholamine concentrations as a result of inhibition of PS leads to the amnesia. Flexner et al.[17] have shown and we have confirmed that CYCLO and ANI reduced the turnover of dopamine and norepinephrine. Quartermain has reported that inhibitors such as diethyldithio-carbamate (DDC) and α-methyl-p-tyrosine (AMPT) caused amnesia.[5] In addition, the monoamine oxidase inhibitors, pheniprazine and pargyline, counteract amnesia.[5] If the above mechanisms are the prevailing mode of action of ANI, then one would anticipate that reducing catecholamine concentration or turnover by catecholamine inhibitors (CAI) would be more effective than ANI in producing amnesia.

ANI, DDC, and tetrabenazine (TBZ) have recently been tested in several experimental paradigms for relative effectiveness on long-term memory with the following results: 1) as shock intensity increased, ANI continued to cause amnesia, while CAIs were effective at only the lowest shock intensity; 2) in a series of multiple injections at 2 h intervals, the effectiveness of ANI increased, whereas additional injections of CAIs were no more effective than saline injections; 3) substitution of a CAI for the second of a series of 3 injections of ANI destroyed the amnesic effect (Flood et al., in preparation). In addition, Squire has also shown that α-AMPT does not have an amnesic effect.[21] We conclude that the critical action of ANI is not its alteration of levels or turn-over of catecholamines.

5) *Does peripheral inhibition prevent an elevation of corticosteroids essential for memory?* Corticosteroids have been shown to block the amnesic effect of CYCLO.[1] This led to the suggestion that CYCLO caused amnesia by lowering corticosteroids rather than by its action of inhibiting PS.[18] Several lines of evidence argue against this, including experiments which showed (1) that emetine blocked ACTH-induced corticosteroidogenesis but did not produce amnesia for an active avoidance task, and (2) that aminoglutethimide which blocks adrenal corticosteroidogenesis, and dexamethasone which blocks pituitary ACTH release, are not amnestic for a passive avoidance task or a visual discrimination task.[27-29] Flood et al. have shown that the depression of corticosterone levels caused by ANI lasted less than 40 min and corticosterone then rebounded to above normal levels even if further injections of ANI were given.[27] However, although two injections of ANI produced amnesia, under the conditions of training used one injection of ANI was not sufficient, as would have been predicted if depression of corticosterone were the mechanism of action of ANI.

6) *Are inhibitors of PS effective in different species?* We were recently surprised to hear the criticism that inhibitors of PS were not effective for a passive avoidance test in rats. This statement is clearly incorrect. Generality

across species has been demonstrated in many instances. For example, in 1972 we reported that CYCLO caused amnesia in rats for a variety of tasks including habituation, left-right discrimination in a Y-maze, reversal bar-pressing for food, and a one-trial passive avoidance task.[7] Schmaltz et al have also shown the amnesic effects of CYCLO with rats in a Y-maze.[30] ANI was also shown to be effective for the step-down passive avoidance test, the only task for which it was employed.[7] Further demonstrations of species generality of effects of PS inhibitors on memory include work with CYCLO and PURO in the goldfish,[11,12] CYCLO and ANI with the chick,[16,31] and CYCLO in drosophila.[32]

THE FUTURE

It seems clear that protein synthesis is an essential for formation of long-term memory traces. Problems for the future include elucidating the steps that control the protein synthesis, and determining what is synthesized. Clearly there must be quantitative differences in the proteins that are synthesized when memory is established, but are there qualitative differences as well? We believe that ultimately one will find highly site-specific synthesis which leads to the modification of neuronal pathways and connections; that is, ultimately anatomical changes will be demonstrated as the critical end product of protein synthesis which has led to the establishment of long-term memory. Neurochemists have a real challenge and a rare opportunity to participate in the unraveling of this age-old problem; we urge them to accept the challenge.

ACKNOWLEDGEMENT

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