

# Effect of Cycloheximide on Protein Synthesis and Memory in Praying Mantis

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JAFFÉ K. *Effect of cycloheximide on protein synthesis and memory in praying mantis.* *PHYSIOL. BEHAV.* 25(3) 367-371, 1980.—Cycloheximide (CXM) was found to be a potent inhibitor of protein synthesis in the nervous ganglia of praying mantis (*Stagmatoptera biocellata*) over a wide concentration range (from 1.75  $\mu\text{g}/\text{animal}$  to 6.6  $\mu\text{g}/\text{animal}$ ) and for a substantial period after injection (more than 80% inhibition for a period of 2-3 hours after injection). When mantises were trained not to attack a mobile star, memory appeared to be disrupted by an injection of CXM administered shortly after training, but after two hours it becomes irresponsive to this agent. This finding is consistent with previous results [5] that reported a connection between formation of a memory trace and the appearance of certain peptides of low molecular weight when mantises were trained not to attack a mobile star. The concepts of "short term memory" and "long term memory" are reexamined in light of this and other works with praying mantis [9].

Learning    Insects    Praying mantis    Cycloheximide

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WHEN praying mantises (*Stagmatoptera biocellata*) are shown a mobile star but prevented from catching it, they learn not to attack the moving object after several trials (no-A learning) [8]. Jaffé and Maldonado [5] have demonstrated a correlation between this learning process and the appearance of certain peptides of low molecular weight. The purpose of the present paper is to study this probable connection between the formation of a memory trace and peptide synthesis using a different approach, i.e. testing the effect of cycloheximide (CXM) on the memory retention.

Studies of this type have been done with insects other than mantis. Brown and Noble [2,3] and Kerkut *et al.* [6] reported that injection of cycloheximide slows up the "leg-raising learning" in cockroaches. However, no data exist about the inhibition of protein synthesis in insects due to CXM administration. Therefore, the present paper includes a previous series of experiments aimed at studying the effect of CXM on protein synthesis in four ganglia of mantis.

## EFFECT OF CYCLOHEXIMIDE ON PROTEIN SYNTHESIS

### METHOD

#### Animals

The animals were adult females (*Stagmatoptera biocellata*) that had reached maturity 20-30 days before the experiments. All animals had been reared in individual cages at a constant temperature of 24°C during the day and 20°C at night, with a relative humidity of 65% and 12 hr of light per day. They were fed four times at 4-day intervals according to the following schedule. The first time, 15 *Sarcophaga* flies; the second time, 10; the third, 5; and the fourth, 2 flies.

#### Reagents

Radioisotope: L [4,5-<sup>3</sup>H(N)] leucine (5 Ci/mmol in 1 ml, New England Nuclear), was used in aq. solution (1:3). Cycloheximide: 3 [2 (3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] Glutarimin (Sigma Chemical Co.), was used in aq. solutions of 0.2%, 0.7%, 1.0% and 2.5%. All solutions of CXM were prepared 30 min before injection.

#### The Mantis-Holder

The mantis-holder used in these experiments has been described elsewhere [12]. The prothorax of each mantis was fixed to a wooden block by means of adhesive tape. Mantis plus block were fitted into a stand and a balance compensated the weight of the animal. A teflon tube 0.3 mm in external diameter and 5 mm long was impaled into the middle dorsal prothorax and sealed around. The dead space of the tube was 5  $\mu\text{l}$ . A 25  $\mu\text{l}$  Hamilton syringe connected to the free extreme of the teflon tube was used for injecting.

#### Procedure and Experimental Design

352 mantises were randomly divided into 22 groups of 16 mantises each, according to the combination of two variables i.e. t: time-interval between injection of CXM and sacrifice (0.5, 1, 3, 6, 8, 16 and 24 hours), and dose of CXM: (0.0, 0.5, 1.75, 2.5 and 6.5  $\mu\text{g}/\text{animal}$ ). Groups of animals injected with distilled water, i.e. [CXM]: 0.0, are called *control groups*. Each mantis received an injection of 25  $\mu\text{l}$  of the corresponding solution, immediately followed by a second injection of 10  $\mu\text{l}$  H<sub>2</sub>O in order to clean the dead space of the cannula. Animals were killed by dropping a mixture of CO<sub>2</sub>-acetone on them, but 15 min before sacrifice they were injected with

TABLE 1  
PERCENTAGE OF PROTEIN SYNTHESIS IN FOUR GANGLIA OF PRAYING MANTIS  
WITH DIFFERENT [CXM]

ganglion	1.75			2.5			6.5			
	t	0.5	1	3	0.5	1	3	0.5	1	3
Brain (B)	14	12	34	12	11*	16	18	6*	13	
Prothoracic (P)	3*	5	7	3*	4	9	5	3*	7	
Mesothoracic (M)	3*	4	12	4*	4*	12	2*	3	7	
Metathoracic (T)	15	11	25	10*	10*	15	5*	10	16	

[CXM]= $\mu$ g of CXM per animal.

t=time-interval in hours between injection of CXM and sacrifice.

\*=minimal value of % of protein synthesis.

25  $\mu$ l of labelled leucine (6.67  $\mu$ Ci/animal). Four nervous ganglia were dissected: "brain" (B), prothoracic ganglion (P), mesothoracic ganglion (M) and metathoracic ganglion (T) "brain" stands in the present paper for a mass of nervous tissue that includes optic lobes, proto- and deutocerebrum. Four ganglia of each kind (B, P, M or T) belonging to the same group were homogenized with 700  $\mu$ l H<sub>2</sub>O. For determining protein content two aliquots of 100  $\mu$ l were taken [7] and the remaining volume was mixed with an equal volume of cold 20% TCA.

After standing in the cold overnight, the precipitate was resuspended with a Vortex mixer and all the volume was passed through a glass fibre paper (Whatman, GF/A) placed on a Millipore Pyrex Microanalysis Filter Holder. The disc was washed four times by filtering 2 ml of cold 5% TCA each time, then removed from the Filter Holder and subjected to an extraction method that was essentially that of Mans and Novelli [14]. Each disc with the protein fraction was placed in a standard glass-counting vial and 2 ml of scintillation mixture was added (0.4% 2,5-diphenyl-oxazole, 0.01% 1,4-bis-2 (5-phenyl)-Oxazolyl). Radioactivity was counted with a model 3310 $\times$ Tri-Carb liquid scintillation spectrometer (Packard Instruments Co.) with 70% efficiency.

#### Percentage of Protein Synthesis

In order to assess the effect of different doses of CXM on different experimental groups, values of percentage of protein synthesis were used. For this purpose the specific radioactivity of protein leucine SRPL (in dpm/mg protein leucine) was evaluated in control and experimental animals. The percentage of protein synthesis was obtained as SRPL of the experimental group $\times$ 100/SRPL of the corresponding control group.

#### RESULTS

Table 1 shows % of protein synthesis with three different doses of CXM at three different times. Maximum inhibition of protein synthesis took place between 0.5 and 1 hour after the drug was injected in all four ganglia.

Figure 1 shows that inhibition of protein synthesis in B does not increase significantly with a dose higher than 1.75  $\mu$ g/animal. Similar curves were obtained for P, M and T. Figure 2 presents % of protein synthesis at different times when mantises received CXM at 1.75  $\mu$ g/animal. Different ganglia evinced different speeds of recovery: B and P recu-

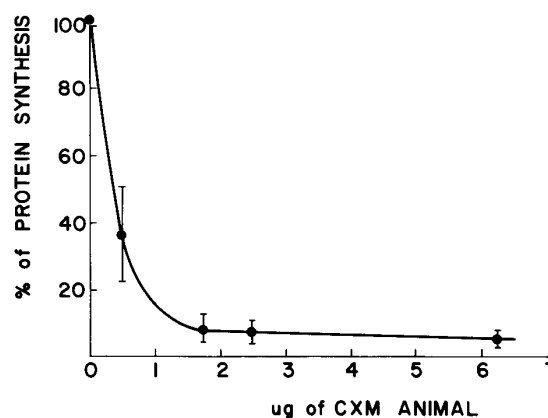


FIG. 1. Percentage of protein synthesis in the brain after 1 hr of an injection expressed as a function of the dose of CXM used. Each point is the mean of four data.

erated more quickly than M and T. After 16 hours the four ganglia had practically recovered all their synthetic ability. During the first 2-3 hr, inhibition turned out to be greater than 80% which is the percentage of inhibition that is considered optimum for disrupting memory retention in mice and goldfish [4]. Similar results were obtained with CXM at 2.5  $\mu$ g/animal.

#### EFFECT OF CYCLOHEXIMIDE ON MEMORY

##### METHOD

##### *Apparatus and Procedure in No-A Training*

Animals, cycloheximide and mantis-holder were as described above. The apparatus has been described elsewhere [8]. It included 48 cages with one mantis placed on a mantis-holder inside each cage. The wall of the cage facing the praying mantis consisted of one piece of glass. In front of it a white screen, 30 cm wide, faced the cage. A black star, 20 mm diameter, fixed into a small cylindrical magnet, was held on the internal face of the white screen by an external magnet. The external magnet was rotated clockwise by the action of a small motor that was placed on a carrier. This carrier, in turn, displaced the figure pulled by the traction of

TABLE 2  
COMPARISONS OF TOTAL NUMBER OF ATTACKS PER SESSION DURING THE NO-A  
EXPERIMENTS (MEANS  $\pm$  SD)

	First training ( <i>t</i> <sub>1</sub> )	Second training ( <i>t</i> <sub>2</sub> )	Third training ( <i>t</i> <sub>3</sub> )	
Control group (C)	15( $\pm$ 12) <i>a</i>	9( $\pm$ 10) <i>b</i>	6( $\pm$ 9) <i>c</i>	
Exp. zero hour group (E-0h)	16( $\pm$ 15) <i>a</i>	15( $\pm$ 14) <i>a</i>	10( $\pm$ 11) <i>b</i>	
Exp. two hours group (E-2h)	13( $\pm$ 11) <i>a</i>	8( $\pm$ 9) <i>b</i>	5( $\pm$ 7) <i>c</i>	
Intragroup comparisons (paired <i>t</i> statistic, $\alpha=0.05$ )				
	$\bar{D}$	SD	<i>t</i>	<i>p</i>
(C) <i>t</i> <sub>1</sub> vs (C) <i>t</i> <sub>2</sub>	6.63	9.21	3.75	<.001
(C) <i>t</i> <sub>2</sub> vs (C) <i>t</i> <sub>3</sub>	7.57	18.33	2.26	.05> <i>p</i> >.
(E-0h) <i>t</i> <sub>1</sub> vs (E-0h) <i>t</i> <sub>2</sub>	0.18	29.0	0.03	NS
(E-0h) <i>t</i> <sub>2</sub> vs (E-0h) <i>t</i> <sub>3</sub>	5.67	8.2	3.77	<.001
(E-2h) <i>t</i> <sub>1</sub> vs (E-2h) <i>t</i> <sub>2</sub>	4.57	6.3	3.92	<.001
(E-2h) <i>t</i> <sub>2</sub> vs (E-2h) <i>t</i> <sub>3</sub>	3.23	5.27	3.36	.005> <i>p</i> >.001
Intergroup comparisons ( <i>t</i> statistic for two means, $\alpha=0.05$ )				

The use of the same letter, i.e. *a*, *b* or *c* following an average stands for means without significant differences. On the other hand, groups with different letters following the averages indicate that the means are statistically different  $p<0.05$ .

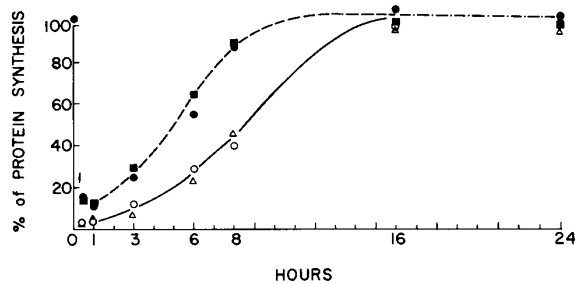


FIG. 2. Time course of percentage of protein synthesis after injection of CXM: 1.75  $\mu$ g/animal, at different time-intervals between injection and sacrifice. Black squares: "brain" (B); black circles: prothorax (P); white circles: metathorax (T); white triangles: mesothorax (M). Each point is the mean of four data.

a motor. A trial consisted in the lateral displacement, throughout the screen in front of a cage, of a clockwise rotating black star which was shown once each 30 sec, i.e. the star was moved at a speed of 1 cm/sec. Every strike of the mantis on the moving object was a frustrated attack, because the frontal glass prevented the animal from catching the figure. The mantis-holder was positioned inside each cage with respect to the center of the figure in such a way that the mantis-star distance represented 75% of the extension of the forelegs. This is the longest distance that can elicit a successful strike on a prey [10]. The number of strikes was computed by simple observation. All mantises carried, fixed with cement on their heads, a fine copper wire with a small ball of tackiwax at one end, that together weighed 100 mg. The rationale for this addition is explained in [11] where it was demonstrated that mantises bearing such a load on their heads showed better retention during no-A training. This is

due to the vibrations of the weight after each attack which works as a negative reinforcement.

#### Experimental Design

A training session *t* consisted of 20 consecutive trials a day with an inter-trial interval of 3 min. Every group received three successive training sessions (*t*<sub>1</sub>, *t*<sub>2</sub> and *t*<sub>3</sub>). After the first training session (*t*<sub>1</sub>) animals were injected with CXM solutions. Thus, sessions *t*<sub>2</sub> and *t*<sub>3</sub> can be considered as sessions for testing and retesting of the retention performances. Ninety mantises were randomly divided into three groups of 30 mantises each. The animals were injected with either distilled water or with a CXM solution (1.75  $\mu$ g of CXM/animal).

The volumes of injection were 35  $\mu$ l for H<sub>2</sub>O; or 25  $\mu$ l for CXM immediately followed by a second injection of 10  $\mu$ l H<sub>2</sub>O in order to clean the cannula. The control group received an injection of H<sub>2</sub>O immediately after the last trial of *t*<sub>1</sub>, the experimental zero hour group received an injection of CXM immediately after the last trial of *t*<sub>1</sub> and the experimental two hours group received an injection of CXM 2 hours after the last trial of *t*<sub>1</sub>. Pilot experiments were done injecting CXM 10 min before the first trial of *t*<sub>1</sub>, but a clear depression in the performance was observed during the first session, i.e. the total number of attacks recorded during *t*<sub>1</sub> was dramatically smaller than those recorded for mantises injected with H<sub>2</sub>O.

#### RESULTS

Table 2 shows results obtained during no-A experiments. Both the control and the experimental two hours groups showed a smaller number of attacks during *t*<sub>2</sub> than were recorded during *t*<sub>1</sub>. The difference in number of attacks is highly significant (*t*-test,  $p<0.01$ ). On the other hand, the experimental zero hour group did not show a significant

difference between  $t1$  and  $t2$ , but a clear-cut decrease in attacks was shown between  $t2$  and  $t3$ .

### DISCUSSION

Cycloheximide was found to be a highly potent inhibitor of cerebral and ganglionic protein synthesis in praying mantis over a wide concentration range, i.e. from 1.75  $\mu\text{g}/\text{animal}$  to 6.6  $\mu\text{g}/\text{animal}$ , and for a substantial period after the injection, i.e. more than 80% inhibition for a period of 2–3 hours after the injection. This period of maximum inhibition proved to be the same for the three doses.

Results indicate that recovery of protein synthesis in both the brain (B) and the prothoracic ganglion (P) after CXM administration was clearly quicker than in the mesothoracic (M) and metathoracic ganglion (T) (Fig. 2). Maldonado *et al.* [12] suggested the existence of a metabolic gradient in the protein synthesis of the different ganglia, where M and T showed both the fastest net synthesis and the fastest net breakdown of the product, whereas B and P showed the slowest net synthesis and the slowest net breakdown. Both findings seem to be congruent, i.e. ganglia with the slowest activity during the protein synthesis (B and P) should be less affected by a CXM pulse than those with a greater activity (M and T).

The results of the behavioral studies showed that mantises learned not to attack the target in that subjects given saline immediately after training or CXM 2 hrs after training made significantly fewer attacks at the 24 hr retention test ( $t2$ ) than they did during original training ( $t1$ ) (15 versus 9 attacks for saline; 13 versus 8 attacks for CXM) and they made even fewer responses on their second retention test given 48 hrs after the original training. The results indicate the subjects given CXM immediately after training failed to show a reduction in the number of attacks when tested 24 hrs later (16 versus 15 attacks). We might account for this amnesic effect if the CXM injected subjects were ill from the after effects of protein synthesis inhibition or if some physiological damage was induced. However, in either case we would expect the subjects to respond less frequently. Since the mantises given CXM immediately after training attacked the target as frequently during training as during the retention test given 24 hrs later, it seems unlikely that illness can account for the amnesic effect. In addition, these subjects were tested again 48 hrs after original training (which was 24 hrs after the first retention test after which no CXM was given) and their number of attacks on the target decreased significantly compared to training or the first testing session. This indicates that the subjects failure to reduce the number of attacks was not the result of some longterm disability which would have prevented learning. By a variety of methods, post-training treatments which impair or improve retention have been found to be time-dependent. That is, as the treatment is administered at successively greater times after training the effectiveness of the agent decreases. CXM was found to have a time-dependent amnesic effect on retention since subjects given CXM immediately after training made significantly more attacks on the target than did subjects given CXM 2 hrs after training. These findings suggest that a) there is a period of memory fixation for no-A that

turns out to be longer than 1 hour, i.e. approximately the total time of a training session of 20 trials, and shorter than three hours, i.e. the interval between the first trial of  $t1$  and the time the animals of the *experimental two hours group* were injected; b) a cerebral and/or ganglionic peptide synthesis is required for the fixation process, i.e. for the "long term" memory storage.

This finding is consistent with that reported by Jaffé and Maldonado [5] demonstrating the appearance of certain peptides of low molecular weight in brains of mantises that had learned not to attack a mobile star. The results of this work together with previous ones done with praying mantis [5,9] suggest that the mnemonic process in this insect is similar to those reported for different vertebrates (1, 4, 15 for example). Thus, the similarity of behavioural and biochemical findings across species suggests that in spite of the different degrees of evolution of the nervous systems, fundamental biochemical mechanisms such as protein synthesis seem to be an essential requirement for the formation of new memories.

Comparisons of this work with one done with the same animal [9] but using a different training method, show that the formation of the so-called "long term memory" (LTM) and the duration of the "short term memory" (STM) is dependent on the training. Using the training 'not to attack a moving star' (No-A), the STM seems to last about one hour and the LTM replaces the STM after this period of time. With the training of 'habituation to the presence of a bird' (No-RD) [9] it was shown that STM lasted up to 6 days and that during this period injections of CXM had no effect on the retention [16]. On the other hand,  $\text{N}_2$ -shock had an amnesic effect on No-RD during this period [9] but did not affect retention of No-A training immediately after one hour of training [16]. This means that No-A learning is fixed as LTM much faster than No-RD learning in the same animal. Thus, we may confirm the conclusions made previously [9,16] that the terms STM and LTM are not appropriate and that the duration of STM or the beginning of LTM are dependent on the training and not on the animal. The two kinds of memory may be more appropriately termed S and I-memory (Sensitive and Insensitive to impairment). These results suggest that I-memory formation after the S-memory is not an automatic process but is subjected to certain conditions. It can be inferred that these conditions are related to a certain probability of the biological usefulness of the learned task and may be genetically predetermined. The No-A learning, i.e. learning to discriminate between potential foods, can be thought of as more useful to the animal and thus is fixed quicker as I-memory than No-RD learning, i.e., learning not to defend itself against a potential danger. This hypothesis presupposes an evolutionary selection of learning abilities related to the specific tasks that will have to be learned rather than a selection of learning abilities per se.

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