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Cycloheximide Produces Attentional Persistence and Slowed Learning in Chickens

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ROGERS, L. J. AND J. M. ANSON. *Cycloheximide produces attentional persistence and slowed learning in chickens.* PHARMAC. BIOCHEM. BEHAV. 9(6) 735–740, 1978.—Cycloheximide has been used previously to demonstrate that long-term memory has a protein substrate. We have shown that it has two additional behavioural effects when it is administered to young chickens. A dose of 10 μg of cycloheximide in 5 μl of normal saline vehicle given to each side of the forebrain on Day 2 of post-hatched life changes attention so that responding to a given stimulus type becomes more persistent. Behavioural testing occurred in the chickens' second week of life. They were given two colours of food, and runs of pecks at each colour were scored. Treated chicks showed less frequent switching from one colour to the other. A 20 μg dose of cycloheximide in 25 μl of saline also produced this state of attentional persistence, and additionally it made the chickens slower at learning a visual discrimination task, a visual habituation task and an auditory habituation task. The paper concludes that the effect on attention is separate from that on learning, and discusses the findings with reference to testosterone's known ability to produce attentional persistence and also with reference to previous research with cycloheximide and long-term memory.

Cycloheximide Attentional persistence Slowed learning Long-term memory Retrieval Visual discrimination learning Chickens

CYCLOHEXIMIDE, a drug which blocks ribosomal protein synthesis, has been used frequently in studies on memory formation, where it is claimed to block consolidation of long term memory [5, 6, 8, 13]. In addition to this property, cycloheximide has been found to produce a permanent reduction in learning rate when it is administered to young chickens [12]. When administered intracranially to a chicken in the first week of life, it not only blocks the formation of long term memory of tasks performed about the time of treatment, but it also alters the brain in such a way that learning on a number of visual and auditory tasks is found to be permanently retarded.

Our previous studies have almost always used a 20 μg dose of cycloheximide dissolved in 25 μl of normal saline administered to each side of the forebrain [12,13]. We therefore considered it important to measure the amount of learning retardation produced by varying the amounts of cycloheximide administered in various volumes of saline, and from these studies an interesting new effect of cycloheximide on behaviour emerged.

Cycloheximide was found to cause the chicken to persist in responding to a given stimulus type once it had begun responding to that stimulus type. This appeared to be remarkably similar to the persistence in responding, or attentional persistence, found previously in male chickens treated with testosterone [4,11]. It was therefore studied in visual search tests designed to measure attentional persistence by scoring runs of pecking on differently coloured food. By appropriate choice of dose followed by measuring both learning rate on a visual discrimination task and attentional persistence, it was possible to separate learning effects from those of attentional persistence.

LEARNING RATES

METHOD

Housing

White leghorn-black australorp cross male chickens were obtained from a commercial hatchery on Day 1 of life. They were housed in groups of 4–6 birds until the evening of Day 3 when they were separated visually from each other. The cages (23×23 cm sq. and 29 cm high) were made of metal with a clear perspex front, through which the chickens could see the daily activity in the laboratory and become familiarized with the experimenter. Constant light and warmth were provided by a 25 W globe suspended above the cage. (For further details see [14]).

Feeding

Food and water were freely available and changed reg-

ularly each day. Chick starter crumbles (Hutmill, Victoria) were spread over a white hand towel on the floor of the cage. This facilitated searching behaviour, as required in testing. The chickens tested for learning on the visual discrimination task were all fed these starter crumbles, which were light brown in colour.

Drug Treatment

Cycloheximide (Sigma, C-6255) was dissolved in 0.9% boiled saline. Intracranial injections of cycloheximide were given freehand into the centre of each forebrain hemisphere of conscious animals. A stopper on the needle prevented penetrated beyond 3 mm below the surface of the head. All drugs were administered on Day 2 of life.

To determine the dose-response relationship for rate of learning on a visual discrimination task 16 groups of 8 birds were injected with the following doses of cycloheximide per hemisphere:-

2.5, 10, 15, 20 μg cycloheximide in 2.5 μl saline
5, 10, 20 μg cycloheximide in 5μl saline
10, 20 μg cycloheximide in 10 μl saline
10, 15, 20, 25 μg cycloheximide in 25 μl saline

Five control groups received only saline at each of the above volumes. Two doses, the 20 μg in 25 μl and the 10 μg in 5 μl, were also tested for visual habituation and auditory habituation learning.

Behavioural Tests

Visual discrimination. The visual discrimination task was used to measure the rate of learning to discriminate grains of chick starter-crumbles from a background of small pebbles stuck down to a clear perspex floor. The test has been described previously in detail [12]. The pebbles covered a range of sizes, shapes and colours, overlapping with those of the grain, but they differed from grain in texture and brightness. Testing was done in the home cage. All scoring was done by eye using a keyboard and an Esterline Angus event recorder.

On Day 9 of life the chickens were deprived of food for 3 hr and then tested for learning. A total of 60 pecks was allowed, and the number of errors, pecks at pebbles, in the last 20 pecks was taken as an index of learning rate. At first they pecked grain and pebbles at random, and in the last 20 pecks controls learnt to find grain and made fewer than 4 erroneous pecks at pebbles.

The animals injected with different amounts of cycloheximide dissolved in various volumes of saline were scored for learning rates on this test. The performance of each cycloheximide-treated group was compared to its control group which received an equal volume of saline.

Visual habituation. When a novel stimulus is placed in the home cage, ongoing behaviour stops and the chicken silently fixates the object. This period of fixation is usually terminated by the chicken's jumping to escape from the stimulus, or occasionally by resuming ongoing behaviour. The time of silent fixation on the fourth presentation of a novel visual stimulus (a torch battery) was taken as a measure of visual habituation rate [14].

Auditory habituation. To test for auditory habituation birds were deprived of food for 4-1/2 hr and then tested while feeding in the home cage. Presentation of a novel sound (banging a lid with a spoon) briefly interrupted feeding. The next stimulus was presented after the bird had established feeding again. Habituation was considered to have occurred when the bird ceased to respond to three consecutive presentations. The number of stimulus presentations needed to produce habituation was scored [14].

RESULTS

Visual discrimination. Chickens given bilateral injections of 20 μg cycloheximide in 25 μl saline or 25 μg cycloheximide in 25 μl saline scored significantly more pecks at pebbles in the last 20 pecks than their appropriate controls ($p=0.002$ in both cases, two-tailed Mann-Whitney U tests). However the performance of all the other groups, which received lower volumes and/or amounts of cycloheximide, was not significantly different from that of the controls. Figure 1 is a three dimensional representation of this data. The actual values for the difference between experimental and control groups in the mean number of errors in the last 20 pecks for each cycloheximide-treated group and its control were as follows:-

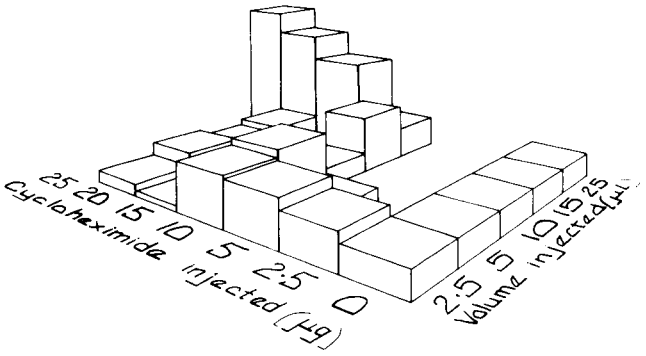

FIG. 1. Dose-response relationship for cycloheximide and slowed learning. This figure is a three dimensional representation of the degree of slowing in learning produced by various doses of cycloheximide. The amount of cycloheximide (in μg) and the volume of saline (in μl) in which it is injected into each side of the forebrain are plotted on the X and Y axes, in the horizontal plane. The amount of slowing in learning rate is represented by a scale plotted in the vertical plane, on the unmarked Z axis. That is, the difference in the mean number of errors in the last 20 pecks between each treated and its control group is plotted, taking a difference of -0.1 as the baseline so that all differences are positive and visual presentation is simplified. Only the 20 μg and 25 μg amounts of cycloheximide in 25 μl of saline produce significant slowing.

For 2.5, 10, 15, and 20 μg cycloheximide given in 2.5 μl saline the differences in mean error scores were 0.6, 1.3, 1.5, -1.2 and -0.7, respectively; for 5, 10 and 20 μg cycloheximide given in 5μl saline they were-0.7, 2.1 and 0.4, respectively; for 10 and 20 μg cycloheximide given in 10 μl saline they were -0.4 and 0; for 10 and 20 μg cycloheximide given in 15 μl saline they were 2.0 and 0.1; and for 10, 15,20 and 25 μg cycloheximide given in 25 μl saline they were 0.3, 4.7, 6.7 and 8.3, respectively.

Some of the cycloheximide treated groups which did not differ from controls in their learning rates were found to peck in longer runs on grain and pebbles. This phenomenon was most clearly demonstrated in the first 20 pecks. The mean run length on pebbles in the first 20 pecks for those groups injected with 5 and 10 μg cycloheximide in 5 μl saline

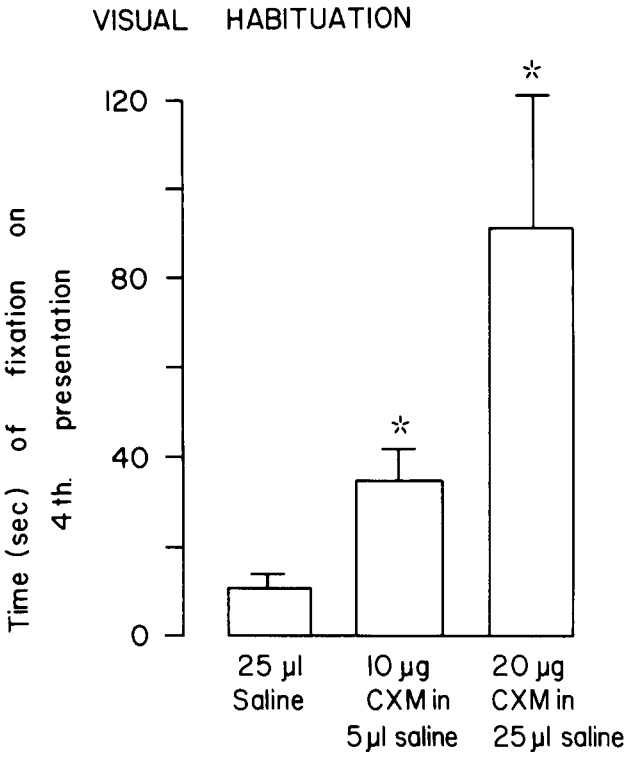

FIG 2. Visual habituation. The number of seconds for which the novel visual stimulus is visually fixated on its fourth presentation is a measure of habituation rate. The larger the score the slower that habituation learning is occurring. The data for 2 doses of cyclohexamide (CXM) and the saline control group is presented as mean values. *indicates a significant difference between the treated group and the control group. p values are given in the text.

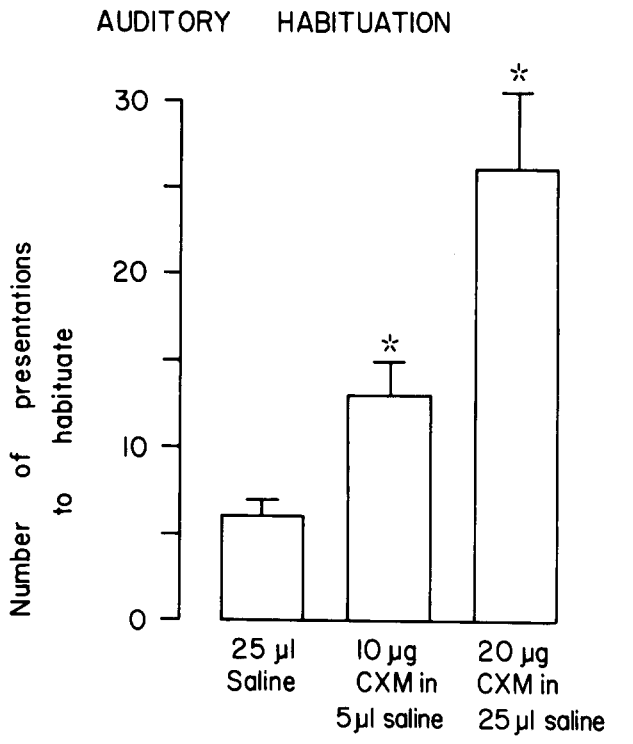

FIG. 3. Auditory habituation. The number of presentations of a novel auditory stimulus until orientation to it ceases is a measure of auditory habituation rate. Mean values for two groups treated with cycloheximide (CXM) and a control saline group are presented. *indicates a significant difference from the control group. p values are given in the text.

were twice as long as those of their equivalent control group ($p=0.022$ and 0.05, respectively, two-tailed U tests). Similar tendencies for longer runs on pebbles were seen in other cycloheximide-treated groups, but this was not significant.

Visual habituation. Both the groups treated with 10 µg cycloheximide in 5 µl saline and the 20 µg cycloheximide in 25 µl saline fixated the novel visual stimulus for longer on the fourth presentation than did controls ($p=0.012$ and $p=0.002$, respectively, two-tailed U tests, see Fig. 2). A graded effect was apparent with the higher dose responding for longer than the group which received 10 µg cycloheximide in 5 µl saline.

Auditory habituation. The groups treated with 10 µg cycloheximide in 5µl saline and 20µg cycloheximide in 25 µl saline required more presentations of the auditory stimulus to habituate than did controls ($p=0.020$ and $p=0.001$, respectively, two-tailed U tests; see Fig. 3). This response was also graded with respect to dose; the group treated with 20 µg cycloheximide in 25 µl saline required significantly more stimuli to habituate than the group treated with 10 µg cycloheximide in 5 µl saline ($p=0.024$, two-tailed U test).

ATTENTIONAL PERSISTENCE

METHOD

Chickens to be tested for "attentional persistence" were fed chick crumbles dyed red. The dyeing procedure was to mix 3.5 g carmine dye with 100 cc water and add this solution to 300 g of chick crumbles. Undyed food was also given to these chickens, and it was prepared by adding the same volume of water to an equivalent amount of chick crumbles without the addition of carmine dye. A preference for red food was developed by feeding these chickens red food continuously, except on Days 5 and 6 when they were given undyed food. (For further details, see [11]).

Drug Treatment

There were three groups each containing 8 chickens. One was injected with 20 µg of cycloheximide in 25 µl of saline in each side of the forebrain, another received 10 µg of cycloheximide in 5 µl of saline in each hemisphere, and the control group received 25 µl of saline into each hemisphere. This treatment was given on Day 2 of life.

Behavioural Testing

To test for attentional persistence the chicken's strategy of search was scored when it was given a choice of two colours of food, red-dyed and undyed, scattered at random on a background of pebbles stuck down to a clear perspex floor. The pebbles were dyed to be a colour similar to that of preferred food (vis., red). Most, if not all, of the learning components were removed from this test by training the chicks to distinguish red grain from pebbles on the day

prior to testing. This occurred on Day 8 of life after 5 hr of food deprivation.

In the first experiment, the three groups (10 μg cycloheximide in 5μl saline, 20 μg cycloheximide in 25 μl saline and 25 μl saline) were trained for 10 min. In the second experiment a group treated with 20 μg cycloheximide in 25 μl saline and a control group was allowed 15 min to learn to distinguish red grain from red pebbles. The ratio of grain to pebbles was maintained at roughly 3:8 by adding more grains as they became depleted.

On Day 9, they were deprived of food for 5 hr and tested on the same floor with red and undyed grains scattered at random over the red pebbles. Full details of the testing procedure have been published previously [11].

Pecks at the three types of stimuli were scored by eye. Only new choices were scored, and not repeated pecks at the same target. Pecking occurred in runs on each type of stimulus; a run being a series of new choices on the same stimulus type uninterrupted by pecks at another stimulus type. Scoring occurring to the end of the run closest to 100 choices. The search strategies used were indicating by calculating the mean run length (MRL), the percentage taken, the longest run and the number of runs on each stimulus type. Longer runs were taken to indicate "attentional persistence," as previously demonstrated in testosterone-treated chickens.

RESULTS

After 10 Min Training on the Previous Day

Table 1 presents the data for scoring the strategies of search adopted on a floor with red food, undyed food and red pebbles. Both the cycloheximide-treated groups pecked significantly more at their preferred food colour (red) than did controls, and their runs on this preferred food were significantly longer. In addition, both treated groups had significantly less runs on undyed food, the nonpreferred colour; the percentage of nonpreferred food taken was less for both treated groups. Compared to the controls, the group treated with 10 μg cycloheximide in 5 μl saline avoided both pebbles and the non-preferred colour of food to a greater extent, and the total number of runs on all three stimulus types was significantly less. That is, the group treated with 10 μg cycloheximide in 5 μl saline was showing less switching of attention, or "attentional persistence."

The group treated with 20 μg cycloheximide in 25 μl saline was showing a similar avoidance of non-preferred food and increased taking of preferred food, but, unlike the group receiving the 10 μg in 5 μl dose of cycloheximide, this group did not avoid pebbles to any greater extent than did controls.

After 15 Min Training on the Previous Day

After extra training to discriminate red grain from red pebbles on the day before testing, a group treated with 20 μg cycloheximide in 25 μl saline performed more like the group above which received 10 μg in 5 μl, in that both pebbles and non-preferred food were now avoided to a greater extent than in the control group. Preferred food was also taken in significantly longer runs by the treated group, as scored by the mean run length, longest run and total number of runs on the preferred food. As found previously for the group treated with 10 μl cycloheximide in 5 μl saline above, the group

TABLE 1

ATTENTIONAL PERSISTENCE AFTER 10 MIN PRETRAINING

Scores for first 100 pecks		25 μl saline	10 μg CXM in 5 μl saline	20 μg CXM in 25 μl saline
Percentage taken	P Food	67	89‡	82*
	NP Food	23	8‡	10‡
	Pebbles	10	2‡	8
Mean run length	P Food	5	20‡	21‡
	NP Food	2	2	1‡
	Pebbles	1	0.8‡	1
Longest run	P Food	14	54‡	39†
	NP Food	5	4	4*
	Pebbles	2	1*	1.7
Total number of runs	P Food	13	5‡	9
	NP Food	11	3‡	4‡
	Pebbles	9	3‡	8

The data in this table represents the pattern of food search adopted in the test with red and yellow food, given after 10 min prior training to discriminate red grain from red pebbles. The mean values (given to the nearest whole number, except for some of the low values for pebbles) are tabulated. The percentage taken was calculated separately for each animal and each stimulus type. Therefore, the means given for percentage taken do not necessarily have to total to 100 percent. The mean run length was calculated for each individual and the overall mean value for the group is tabulated. Two-tailed Mann-Whitney U tests have been applied to the individual scores between each treated group and the saline control group. The significant values are indicated thus: *means $0.01<p<0.05$; †means $0.005<p<0.01$; ‡means $0.000<p<0.005$.

TABLE 2
ATTENTIONAL PERSISTENCE AFTER 15 MIN PRETRAINING

Scores for first 100 pecks		25 μl saline	20 μg CXM in 25 μl saline
Percentage taken	P Food	80	88*
	NP Food	16	11
	Pebbles	3.8	1.1
Mean run length	P Food	22	49†
	NP Food	3	4
	Pebbles	1.1	0.6†
Longest run	P Food	39	79§
	NP Food	6	7
	Pebbles	1.4	0.6†
Total number of runs	P Food	8	3§
	NP Food	6	2†
	Pebbles	3.9	1.2‡

The pattern of search after 15 min pretraining to discriminate red grain from red pebbles is tabulated as mean values, as in Table 1, but with the following indications of signficant p values: *means $p=0.075$; †means $0.01<p<0.025$; ‡means $p=0.01$; §means $0.001<p<0.01$.

treated with 20 μg cycloheximide in 25 μl saline now had significantly fewer runs on all three stimulus types.

DISCUSSION

Learning rate on the visual discrimination task is made slower by injecting either 20 μg or 25 μg of cycloheximide dissolved in 25 μl of saline into each hemisphere of the chicken forebrain. These are the only two combinations of amount of drug with volume of vehicle which produce the effect. Reduction of either the amount of cycloheximide or volume of saline below these amounts fails to produce slower visual discrimination. It has been shown that the 20 μg in 25 μl dose inhibits ribosomal protein synthesis throughout the chicken forebrain [15]. Smaller doses inhibit protein synthesis throughout the same area of the forebrain, but the percentage inhibition is much smaller [15]. This suggests that at least 80% inhibition is necessary to cause retarded visual discrimination learning.

Although the groups treated with 10μg and 5μg of cycloheximide in 5μl of saline learnt at the same rate as controls, they adopted a different searching strategy in the first 20 pecks of the visual discrimination test. In these groups, the mean length of runs of pecks at pebbles was significantly longer than that scored for controls, even though the total number of pecks delivered to pebbles in the first 20 pecks was the same as for controls. The cycloheximide-treated groups were switching between grain and pebbles less often. This appeared to be an effect on searching behaviour remarkably similar to that reported previously to be present in male chickens treated with testosterone and attributed to an alteration in attention [1, 3, 4, 11]. Longer runs of pecking on each stimulus with less frequent switching between all the available stimulus types is said to indicate "attentional persistence" [2] and it can best be demonstrated on a searching task for red and yellow food scattered on a background of red pebbles.

After 10 min of training to discriminate red grain from red pebbles on the previous day the chicks treated with 10 μg of cycloheximide in 5 μl of saline pecked in longer runs on their preferred food colour (red) and avoided non-preferred food and pebbles to a greater extent than did controls. This is the same sort of attentional persistence seen previously to be produced by testosterone treatment of chickens [4,14].

The group treated with 20 μg of cycloheximide in 25 μl of saline also pecked in longer runs on the preferred food and avoided non-preferred food to a significantly greater extent than did controls. This in itself is indicative of attentional persistence, but the data for this group was confounded to some extent by there being more pecks at pebbles than found in the other treated group. This was considered to be a problem produced by the fact that the group treated with 20 μg of cycloheximide in 25 μl of saline is slower to learn and may not have been given sufficient time to learn to discriminate red food from red pebbles on the previous day [12].

Allowing an extra 5 min training to discriminate red food from red pebbles confirmed this supposition. After longer training with red food and red pebbles on the previous day the group treated with 20 μg of cycloheximide in 25 μl of saline had a searching pattern identical to that found in the group treated with 10 μg of cycloheximide in 5 μl of saline. Both the treated groups now pecked in longer runs on preferred food and avoided non-preferred food and pebbles to a greater extent than controls. That is, attentional persistence was demonstrated at both doses, one which also caused slow visual discrimination learning and one which did not.

Since it is possible to remove most obvious learning factors from the task for measuring attentional persistence, and since doses of cycloheximide which fail to alter learning rate on the visual discrimination task are found to cause attentional persistence, we can deduce that cycloheximide's effect on attention does not require a concomitant defect in visual discrimination learning ability in order to be expressed. One cannot, however, say that the defect in visual discrimination learning is independent from cycloheximide's effect on attention, because the dose effective in producing slowed learning also causes attentional persistence.

Both doses of cycloheximide caused significant slowing in the rates of visual and auditory habiutation, but 10 μg of the drug in 5 μl of saline was much less effective than 20 μg in 25 μl of saline. It is therefore impossible to completely separate attentional persistence from slowed habituation rate but attentional persistence is unlikely to be the sole factor contributing to the scores on these tests.

Cycloheximide treatment of young chickens therefore affects several neural mechanisms which have wide ranging effects on behavior. Many researchers have used cycloheximide to demonstrate that consolidation of memory requires protein synthesis [5, 6, 8, 13]. We have found that it produces a permanent retardation in learning rate on both visual and auditory tasks [14]. And now, it has been shown to produce changes in attention which persist for a considerable time after treatment.

Since the attentional effect is separable from the effect on learning rate, we consider that these should be considered as two separate behavioural effects, at least at this point in our knowledge. However, it would be premature to say that the claimed ability of cycloheximide to block memory consolidation is yet a further, discrete behavioral effect of the drug. Changes in motivation or attention during retention tests for memory are always a problem, and now that we know that cycloheximide changes attention span, these studies on

memory need to be re-examined, particularly where young chickens have been used [8,13]. Indeed, Quartermain [9,10] has found that long-term memory returns after cycloheximide treatment, either spontaneously or after a reminder cue is presented. Consequently, he suggested that cycloheximide was causing blockage of retrieval and not memory consolidation. Our results could be in line with this possibility.

The final point of interest is that cycloheximide, a blocker of protein synthesis, and testosterone, an anabolic steroid, both produce attentional persistence. An explanation for this at the biochemical level is unclear. However, testosterone, at certain doses, is known to have an anabolic effect on some tissues and a simultaneous catabolic effect on others [7]. It is therefore possible that, while testosterone stimulates protein synthesis in some areas of the brain, it lowers it in others and, in so doing, causes some effects similar to those of cycloheximide. Alternatively, cycloheximide may, in some way, be stimulating the release of endogenous testosterone, or one of the other steroids known to produce attentional persistence [14].

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