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Cycloheximide, amino acid incorporation and learning in the isolated cockroach ganglion

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A NUMBER of recent investigations on vertebrates have suggested a role for protein synthesis in learning. The vast complexity of the central nervous system in these animals, with its array of sensory and motor systems, has raised difficulties in identifying the specific mechanisms involved in the acquisition and retention of a learning task. In search of more discrete preparations, Horridge[1] has found that the ventral ganglia, in a headless cockroach, are able to associate repeated punishment by electric shock with leg position. Further studies[2] have revealed that even a single isolated cockroach ganglion can support avoidance learning. By utilizing this latter preparation, we have recently reported[3] that the application of 375 μg/ml of cycloheximide, an inhibitor of peptide bond synthesis, results in a decrement in both initial learning and in subsequent relearning, but not in a decline in activity or responsiveness to shock. The present study describes the extent of inhibition of amino acid incorporation in the isolated cockroach ganglion correlated with learning deficits after cycloheximide treatment.

Paired adult male cockroaches (*Periplaneta americana*) were matched for size and for generally healthy appearance and were run simultaneously. Prothoracic ganglion preparations were obtained by the methods of Eisenstein and Cohen.[2] Methods used to assess learning of conditioned shock avoidance have been previously described.[3] For the radioactive studies, the tip of a vertically suspended 25-μl microcapillary tube filled with an insect saline solution, containing 375 μg/ml of cyclomeximide and 9·1 μc of 4,5 ^{3}H-D, L-lysine (2·9 c/mM), was applied to the ventral surface of the experimental ganglion and the solution was permitted to diffuse out. An equal volume of saline containing 9·1 μc of ^{3}H-lysine was simultaneously applied to the control ganglion. Sixty min after the onset of application, each ganglion was dissected free of adjacent tissues and homogenized in 1·0 ml of cold saline. The homogenate was transferred to a 12-ml centrifuge tube with the aid of 2 ml of cold saline followed by a 3-ml wash of 10% trichloroacetic acid. The entire mixture was agitated for 30 sec in a Deluxe mixer followed by centrifugation at 2000 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 2 ml of chloroform–methanol (1:1). After centrifugation for 5 min at 2000 *g*, the supernatant was discarded and the pellet was subjected to a second identical chloroform–methanol extraction. To the resulting pellet, 0·5 ml of 1 N NaOH was added and the mixture was placed in a 100° water bath for 10 min. The resulting solution was cooled and centrifuged to remove undissolved material. A 50-μl aliquot of the supernatant was analyzed for protein by the procedure of Lowry *et al.*[4] and a second similar aliquot was added to a hyamine–toluene mixture and its radioactivity determined in a Packard liquid scintillation counter. The specific activity of each ganglion was then defined as cpm/μg protein and specific activity ratios for matched experimental to control pairs were determined.

Fig. 1 shows the dose–response relationship between cycloheximide concentration and inhibition of learning. The data indicate that ganglia exposed to increasing concentrations of cycloheximide require more time to achieve criterion on initial learning of the conditioned avoidance task; i.e. higher concentrations of drug led to greater inhibition of initial learning.

The effects of cycloheximide on ^{3}H-lysine incorporation into ganglionic proteins* are presented in Fig. 2 and are expressed as per cent ratio of specific activities of matched experimental to control pairs. The data show that exposure to increasing concentrations of drug resulted in decreased incorporation of labeled amino acid into ganglionic proteins. A calculation of the correlation coefficient[5] between per cent ratio of specific activities and minutes to criterion corresponding to cycloheximide doses of 100, 300, 375 and 500 μg/ml yielded $r = -0{\cdot}94$. This implies a strong relationship between decreased levels of amino acid incorporation and increased times required to achieve learning criterion; i.e. as protein synthesis declines, so does learning.

* The protein content (mean $\pm$ S.E.) for 30 saline-treated ganglia, including 10 from a preliminary study, is 84·4 $\pm$ 5·6 μg/ganglion. Wet weight, as determined on a Cahn Electrobalance, is 1170 $\pm$ 80 μg/ganglion.

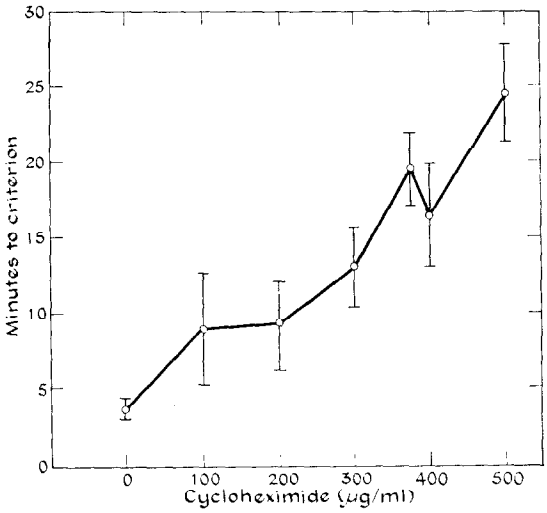

FIG. 1. Cycloheximide and learning in isolated prothoracic ganglion preparations of *Periplaneta americana*. Values represent mean ± S.E.; n = 17 pairs for the 0 and the 375 (μg cycloheximide/ml) points and n = 10 pairs for the 100, 200, 300, 400 and 500 points.

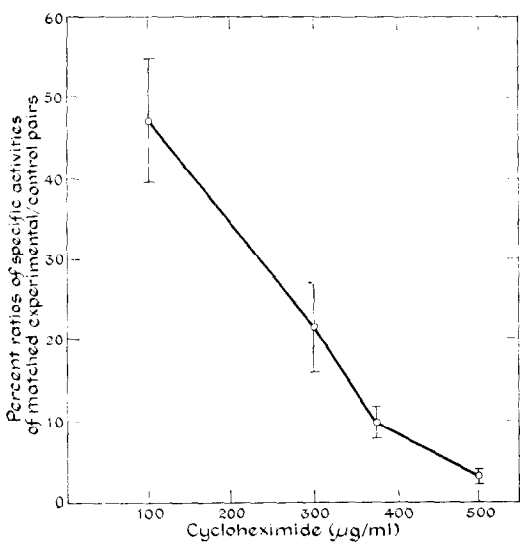

FIG. 2. Cycloheximide and ^{3}H-lysine incorporation into proteins of isolated prothoracic ganglion preparations of *P. americana*. Values represent mean ± S.E.; n = 10 pairs for the 375 (μg cycloheximide/ml) points and n = 5 pairs for the 100, 300 and 500 points.

Shock responsiveness and activity indices, as described in a previous study,[3] were applied to assess possible toxic effects of cycloheximide on the behavior of groups of learning preparations. A plot of median number of shocks per shock period versus minute of training is shown in Fig. 3. The data indicate that clear-cut toxic effects appeared first in ganglia exposed to 500 μg/ml of cycloheximide. The latter group of preparations showed a markedly decreased responsiveness to shock in that its

ganglia tended to receive many more continuous shocks than ganglia in other groups prior to raising their legs to effect shock offset by breaking the circuit. This same group also shows the lowest activity of any of the groups tested, i.e. it made the fewest movements (Fig. 4). Groups exposed to 100, 200, 300, 375 and 400 μg/ml of the drug show activity and responsiveness similar to those of the control groups (Figs. 3 and 4).

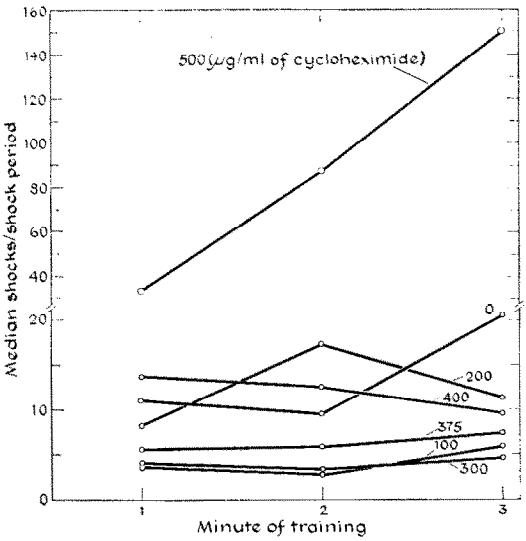

FIG. 3. Cycloheximide and shock responsiveness of isolated prothoracic ganglion preparations of *P. americana*. Values represent mean ± S.E.; n ± 17 pairs for the 0 and 375 (μg cycloheximide/ml) points and n = 10 pairs for the 100, 200, 300, 400 and 500 points.

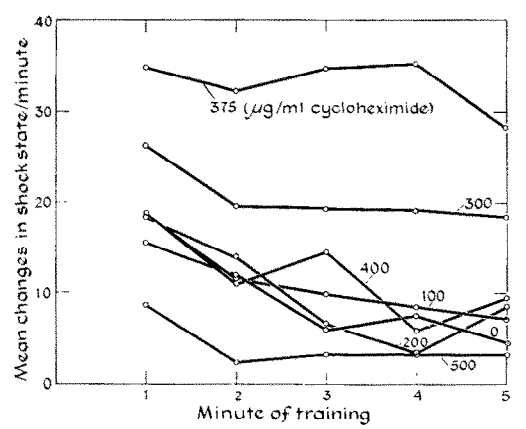

FIG. 4. Cycloheximide and activity of isolated prothoracic ganglion preparations of *P. americana*. Values represent mean ± S.E.; n = 17 pairs for the 0 and 375 (μg cycloheximide/ml) points and n = 10 pairs for the 100, 200, 300, 400 and 500 points.

Previous studies have shown that cycloheximide affects amino acid incorporation in a variety of systems. Young *et al.*,[6] after the i.p. injection of 50 mg/kg of cycloheximide, found 90 per cent inhibition of ^{14}C-amino acid incorporation into rabbit liver protein. Jondorf *et al.*[7] observed 41–95 per cent, inhibition of hepatic microsomal enzyme activity in rats after the i.p. injection of 1 mg/kg of the drug

while Gorski and Axman[8] reported that a 10 mg/kg (i.p.) injection of cycloheximide resulted in 94 per cent inhibition of ^{14}C-amino acid incorporation in rat uterine protein. In the present study, the method of introducing the drug was by topical application rather than by i.p. injection; hence there is no standard of comparison with previous studies for the amount of cycloheximide employed. However, the extent of inhibition of amino acid incorporation in the present study and in those cited above is similar.

The purpose of employing cycloheximide in learning studies by utilizing the cockroach ganglion preparation was to correlate changes in learning with inhibition of protein synthesis. Data presented in several previous studies implied that cycloheximide might be a suitable agent. Ennis and Lubin[9] showed that: (1) the drug suppressed protein synthesis in intact mammalian cells before any effect on RNA synthesis appears; (2) inhibition of protein synthesis was reversible; (3) transfer of amino acid from sRNA to polypeptide was inhibited; and (4) release of nascent polypeptide chains was not accelerated. Cohen and Ervin[10] showed that, unlike puromycin, cycloheximide injected intracerebrally did not result in abnormal hippocampal electrical activity in mice.

The treatment of cockroach ganglion preparations in both the previously cited[3] and the present learning experiments was identical to the treatment of ganglia in the amino acid incorporation experiment up to the point of dissecting out the ganglia, except that no radioactive amino acids were applied in the behavioral study. Thus, we are led to conclude that in the cockroach ganglion system, learning deficits accompany the inhibition of ganglionic protein synthesis after cycloheximide treatment at dosages causing no toxic effects demonstrable on our behavioral indices. Whether such learning deficits are in fact due to more subtle toxic effects of cycloheximide or to other drug effects not causally related to protein synthesis inhibition remains to be demonstrated.

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