

## A CHEMICAL CORRELATE OF LEARNING IN A PRAYING MANTIS

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**Abstract**—Bidimensional and unidimensional maps of amine-containing components extracted from brains of the praying mantis (*Stagmatoptera biocellata*) were obtained using high-voltage electrophoresis and chromatography, and high-voltage electrophoresis alone.

Bidimensional maps from control insects, i.e. animals that did not receive training, showed four distinctive spots and one less intense spot (number 5). On the other hand, bidimensional maps from trained animals, i.e. mantids that were trained not to attack a moving star, showed the same spots 1-4, plus an intense spot (number 5) and an extra component (number 6).

Unidimensional maps from brains of mantids that were trained not to attack a moving star ('star-group') showed two extra components in comparison with maps from the control insects. On the other hand, when mantids received training similar to that of the star-group, but using a fly that could not be caught as a stimulus, instead of a mobile star, they did not learn and their maps were similar to those from control mantids.

The techniques used in this paper to obtain the maps suggest that they are maps of peptides of low molecular weight. The possible correlation between the appearance of extra spots in the maps and a learning process is discussed.

### INTRODUCTION

CONSOLIDATION into long-term memory is thought to require a stable process and this assumption has prompted the search for chemical correlates of learning. Thus, a connection between specific proteins or peptides of low molecular weight, and the memory trace, has often been claimed in vertebrates (HYDÉN and LANGE, 1970; POHLE and MATTHIES, 1974).

Similar studies have been carried out with insects. The results of BROWN and NOBLE (1967, 1968) and KERKUT *et al.* (1970b) indicate that injection of cycloheximide or actinomycin D slows up the 'leg-raising learning' in cockroaches. Since these drugs are inhibitors of protein synthesis, a connection between protein synthesis and formation of the memory trace was also suggested here. Other findings proved to be consistent with such a suggestion. A double labelling technique indicated that trained cockroaches incorporate more labelled uridine into their ganglionic RNA than the control or resting animals, and this result was confirmed by autoradiographic studies on metathoracic ganglia (KERKUT *et al.*, 1970b). Incorporation of labelled amino acids into a TCA precipitable fraction from cockroach ganglia was found to take place during learning (KERKUT *et al.*, 1970a). A double labelling technique indicated that three protein fractions show such increased incorporation (KERKUT *et al.*, 1972).

Amino acid and protein metabolism have been studied in the nervous system of the praying mantis (MALDONADO *et al.*, 1976a). On the basis of these studies, MALDONADO *et al.* (1976b) investigated whether learning not to attack a mobile star (no-A

training, MALDONADO, 1972) altered the protein metabolism in the ganglia and whether some specific protein fractions were involved. Unlike the findings of KERKUT *et al.* (1972), experiments using double labelling and electrophoresis indicated that changes after no-A training involve all the proteins, and are not restricted to one or a few protein species. Taking into account both these results and other findings that suggest a possible role of small peptides in memory formation (UNGAR and RUSSELL, 1972), experiments reported here were aimed at testing a correlation between the no-A learning in mantids and the appearance of specific peptides of low molecular weight.

### MATERIALS AND METHODS

#### *Animals*

The animals were adult females *Stagmatoptera biocellata* that had reached maturity 15-20 days before the experiments. All animals had been reared in individual cages at a constant temperature of 29°C during the day and 24°C during the night with a relative humidity of 65% and 12 hr light per day. After the last ecdysis 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.

#### *The mantis-holder*

The mantis-holder used in these experiments has been described elsewhere (MALDONADO *et al.*, 1976a). The prothorax of each mantis was fixed to a wooden block by means of adhesive tape. The insect and block were

fitted into a stand and a balance used to compensate for the weight of the animal.

#### Apparatus, procedure and experimental design

The apparatus for no-A training has been described elsewhere (MALDONADO, 1972). 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 a piece of glass. In front of it a 30 cm wide white screen faced the cages. A black star, 20 mm dia. or a live fly (*Sarcophaga* sp.), fixed on to a small cylindrical magnet, was held on the internal face of the white screen by an external magnet which was rotated clockwise by the action of a small motor placed on a carrier. This carrier, in turn, displaced the figure or the fly pulled by the traction of a motor. A trial consisted of the lateral displacement across the screen in front of a cage, of the black star or the fly, which was shown once during 30 sec, i.e. the star or the fly was moved at a speed of 1 cm/sec. Every strike of the mantis towards the moving object was a frustrated attack, because the frontal glass prevented the animal from catching it. The mantis-holder was positioned inside each cage with respect to the centre of the star or the fly in such a way that the mantis-stimulus distance represented 75% of the extension of the forelegs, i.e. the longest distance that can elicit a successful strike (MALDONADO *et al.*, 1967). The number of strikes was observed visually and recorded. All trained insects carried a fine copper wire, fixed to their heads with cement, with a small ball of tackiwax at one end, that together weighed 100 mg. The rationale for this addition is explained in MALDONADO and TABLANTE (1975) where it was demonstrated that mantids bearing such a load on their heads show better retention during no-A training.

#### Experimental design

There were four different groups of mantids, i.e. star-group 250, star-group 30, fly-group 30 and control-group. The star-group 250 (stimulus: black

star; total number of trials: 250) included 182 mantids that received three training sessions ( $e_1$ ,  $e_2$  and  $e_3$ ) with intervals of 1 day between sessions.  $e_1$  and  $e_2$  consisted of three blocks of 40 trials with intervals of 1 hr between blocks.  $e_3$  consisted of only one block of 10 trials. The star-group 30 (stimulus: black star; total number of trials: 30) included 99 animals that received two training sessions ( $e_1$  and  $e_2$ ) with intervals of 1 day between sessions.  $e_1$  consisted of only one block of 20 trials and  $e_2$  of 10 trials. The fly-group 30 (stimulus: a live *Sarcophaga*; total number of trials: 30) included 52 animals that received training organized in the same way as star-group 30. Intertrial intervals were 4 min for all the groups. Before starting  $e_1$ , the insects of all three experimental groups were kept four days in the apparatus, fixed to the mantis-holder. Immediately after the last trial, they were killed by dropping on them a slurry of solid  $\text{CO}_2$  in acetone. The control-group included 89 mantids that were sacrificed after being kept in the apparatus, fixed on the mantis-holders, for 7 days, but without being trained. The vivarium-group included 52 mantids that were sacrificed 15–20 days after reaching maturity, but without being mounted on the mantis-holder or placed in the apparatus.

#### Preparation of the brain extracts and gel filtration

Brains were dissected according to the method used by MALDONADO *et al.* (1976a). The 'brain' stands in this paper for a mass of nervous tissue that includes optic lobes, proto- and deuto-cerebrum. Brains from one experimental group of mantids were pooled and homogenized in 1 ml  $\text{H}_2\text{O}$  in a glass homogenizer with a motor driven Teflon pestle. The homogenate was centrifuged at 80,000 *g* for 1 hr and the supernatant was chromatographed through a column of molecular exclusion Sephadex G-75 (1.6 × 32 cm). Elution was performed with distilled water and the OD of the fractions was measured at 280 nm. During pilot experiments fractions of 4 ml were collected from supernatants of the homogenates of 14 brains. Two

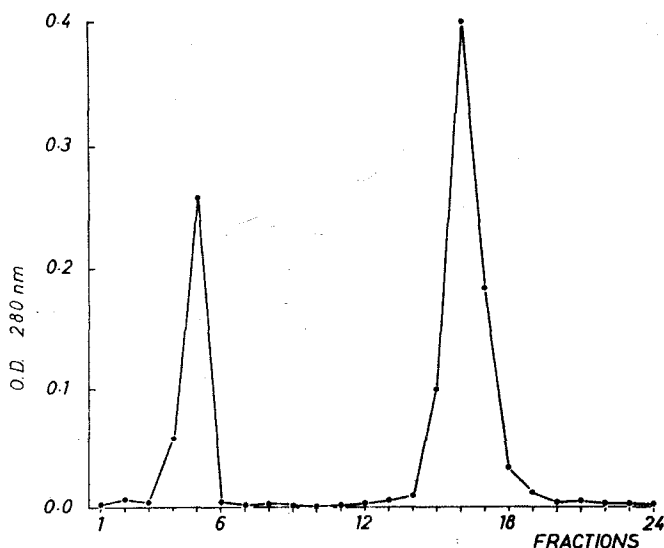


Fig. 1. Elution profile of brain extracts that were chromatographed through a column of Sephadex G-75. Ordinate: optical density at 280 nm. Abscissa: fractions of 4 ml each collected from supernatants of the homogenates of 14 brains.

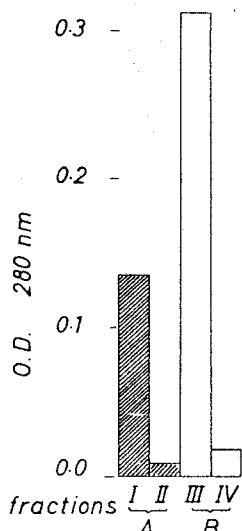


Fig. 2. Values of optical density corresponding to four fractions of 25 ml each, collected after chromatography in a column of Sephadex G-75. Extracts from 14 brains. White bars: fractions III+IV (B) used for obtaining the fingerprints. Diagonal bars: fractions I+II (A) were discarded.

clear-cut peaks were obtained in all elution profiles (Fig. 1). Therefore, final experiments were carried out collecting only four successive fractions of 25 ml from pools of 14–15 brains (Fig. 2). The quotient between O.D. values of I+II fractions, i.e. A fraction, and that of III+IV fractions, i.e. B fractions, was around 0.43–0.44. O.D. values of B varied between 0.3 and 0.35.

#### Electrophoretic and fingerprint maps

During the first series of experiments every B fraction was lyophilized, and dissolved in 100  $\mu$ l of pyridine-acetate buffer (pH 6.4). High-voltage

electrophoresis (Savant Instruments Inc.) was performed at 2400 V for 30 min in the same buffer using 3 MM Whatman paper. Then, an ascending chromatogram was run for 20 hr, at right angles to the direction of electrophoresis, using a mixture of pyridine–iso-amyl alcohol–water (3:3:3.5, v/v). The amine-containing components thus separated were visualized with ninhydrin or fluorescamine (MENDEZ and LAI, 1975). During the second series of experiments chromatography was omitted.

## RESULTS

### (1) Performance of the trained groups

Table 1 shows the means of number of attacks per animal during the first ten trials of every training session. Results corresponding to each experimental group are presented in two sub-groups, i.e. attackers and sub-attackers. Attackers stand for those mantids that attacked more than three times in the first ten trials of  $e_1$ . Sub-attackers are those mantids that attacked three times or less than three times during the first ten trials of  $e_1$ . Inspection of Table 1 reveals that both star-groups contain a greater number of sub-attackers than the fly-group, i.e. a live fly is more attractive than a mobile star. Mean values corresponding to attackers of both star-groups show a clear-cut fall from  $e_1$  to  $e_2$ . On the other hand, attackers in the fly-group do not show a significant  $e_1 - e_2$  difference. As regards sub-attackers, only those in the star-group 250, i.e. those that received 120 trials during  $e_1$ , exhibit an  $e_1 - e_2$  difference that reaches significance.

### (2) First series of experiments

Figure 3 shows maps obtained from pools of 14 brains corresponding to the vivarium-group, the control-group, attackers of the star-group 250 and sub-attackers of the same group. Maps from mantids

Table 1. Comparisons of number of attacks per animal (means  $\pm$  S.D.) during the first ten trials of each session

Group	<i>n</i>	$e_1$	$e_2$	$D_{1-2}$	<i>t</i>	<i>P</i>	$e_3$	$D_{1-3}$	<i>t</i>	<i>P</i>
Star-group 250 Attackers	100	13.45 ( $\pm 9.08$ )	5.20 ( $\pm 7.25$ )	+8.25 ( $\pm 8.74$ )	9.44	<0.001	2.30 ( $\pm 3.20$ )	+11.15 ( $\pm 8.75$ )	12.74	<0.001
Star-group 250 Sub-attackers	82	1.35 ( $\pm 1.00$ )	0.52 ( $\pm 1.05$ )	+0.83 ( $\pm 1.05$ )	7.16	<0.001	0.40 ( $\pm 1.20$ )	+0.95 ( $\pm 1.20$ )	7.17	<0.001
Star-group 30 Attackers	51	13.47 ( $\pm 9.42$ )	7.75 ( $\pm 7.23$ )	+5.72 ( $\pm 6.17$ )	6.62	<0.001				
Star-group 30 Sub-attackers	48	0.98 ( $\pm 0.94$ )	0.90 ( $\pm 2.35$ )	+0.08 ( $\pm 1.14$ )	0.49	NS				
Fly-group 30 Attackers	44	13.10 ( $\pm 6.41$ )	12.39 ( $\pm 9.14$ )	+0.72 ( $\pm 6.27$ )	0.76	NS				
Fly-group 30 Sub-attackers	8	0.75 ( $\pm 1.04$ )	0.88 ( $\pm 1.46$ )	-0.13 ( $\pm 0.99$ )	0.37	NS				

*n* = number of animals per subgroup.

$e_n$  = training session.

$D_{1-2}$  = mean of paired differences between  $e_1$  and  $e_2$ .

$D_{1-3}$  = mean of paired differences between  $e_1$  and  $e_3$ .

*P* = probability according to paired *t* statistic,  $\alpha = 0.05$ .

N.S. = no significance difference.

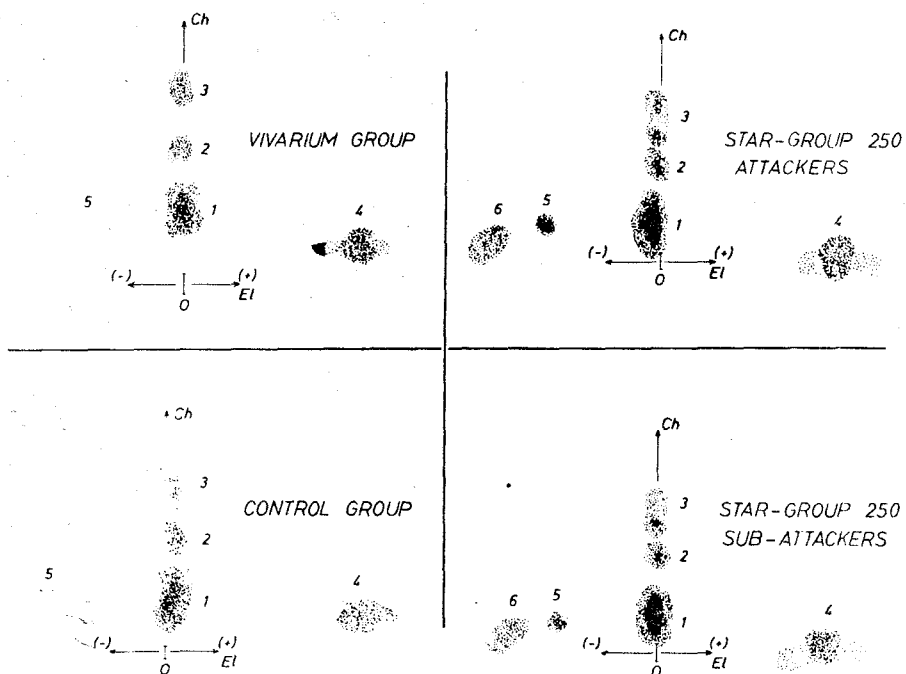


Fig. 3. Maps of the amine-containing spots corresponding to brains extracted from four different groups of mantis. Directions of chromatography (Ch), electrophoresis (El), and the point of sample application (0) are indicated. The four maps were stained with ninhydrin.

that did not receive training, i.e. vivarium and control groups, showed four quite distinctive spots (number 1–4), and one less intense spot (number 5). In other maps from untrained mantids spot number 5 was completely absent. Maps corresponding to mantids that received training, i.e. those from the star-group 250, also showed spots 1–4 and an intense spot number 5, with an additional spot number 6. Similar results were obtained in a total of four maps from the vivarium group, three from control group, four from attackers of the star-group 250 and two maps from sub-attackers of the same group. The intensities of spots 1, 2, 3 and 4 varied in different maps without correlation with the experimental conditions. Maps stained with ninhydrin or fluorescamine showed the same number and bidimensional distribution of spots.

### (3) Second series of experiments

17 electrophoretograms were run from pools of 14, 15 and 18 brains, 2–3 runs for each of the six experimental groups discussed below.

Figures 4 and 5 are examples of such electrophoretic maps. Figure 4 shows the components that migrated towards the cathode and Fig. 5 those that migrated towards the anode. All the maps reveal similar components on the positive side (Fig. 5), i.e. three quite distinctive spots ( $-2$ ,  $-3$  and  $-4$ ) and a tenuous spot ( $-1$ ) that sometimes cannot be distinguished. On the other hand, components on the negative side (Fig. 4) vary in accordance with the experimental conditions. Examination of all the maps corresponding to the control group, fly-group 30 (attackers) and star-group 30 (sub-attackers) reveals only one distinctive spot ( $+1$ ), whereas in all the maps corresponding to the star-group 30 (attackers), star-group 250 (attackers) and star-group 250 (sub-attackers) there are three distinctive spots ( $+1$ ,  $+2$

and  $+3$ ). In summary, maps obtained from brains of mantids that had learnt show two extra-components on the negative side, i.e.  $+2$  and  $+3$ .

## DISCUSSION

The methods used in the present work, for the isolation and qualitative identification of ninhydrin-positive material extracted from mantis brains, allows us to tentatively identify this material as a mixture of peptides with low molecular weight.

Bidimensional maps from the first series of experiments showed clear differences between untrained and trained mantids. Only the latter maps had an intense spot number 5 and an additional spot number 6 (Fig. 3). Furthermore, groups that effectively learnt but that sharply differed in activity, i.e. attackers and sub-attackers of the star-group 250, showed similar fingerprints. On the other hand, the close similarity between maps from the vivarium and control groups eliminates any explanation based on differences in management, since mantids of the vivarium group were sacrificed without having stayed in the apparatus whereas those of the control group received treatment as similar as possible to the trained mantids. These first findings suggest that some peptides of low molecular weight could be involved in no-A learning. However, no unequivocal relationship between the extra spots in the maps and the memory trace could be established with certainty from the bidimensional maps. In fact, trained mantids differed from untrained mantids in more than one way: they were trained not to attack a black figure and they also suffered a punishment at every strike, i.e. a wide oscillation of the head caused by the wire fixed to the epicranial sclerite. Furthermore, trained and

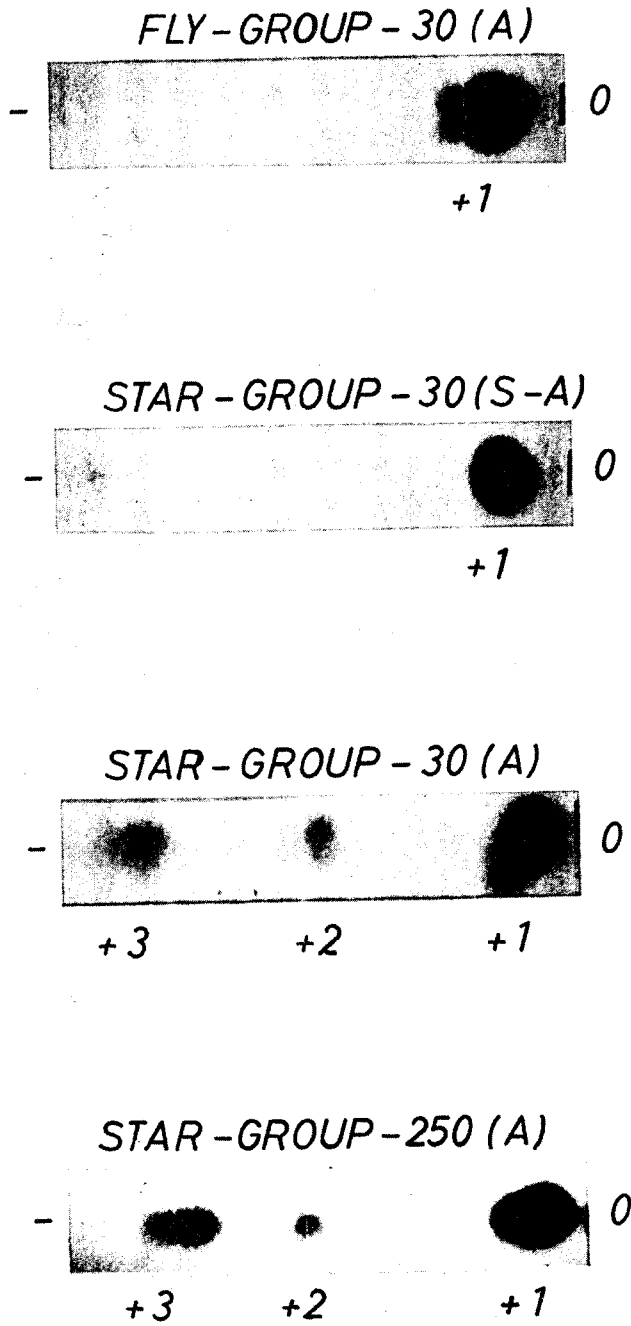


Fig. 4. Cathodic side of electrophoretic maps. (A) = attackers; (S-A) = sub-attackers; 0 = : point of sample application; - cathodic end.

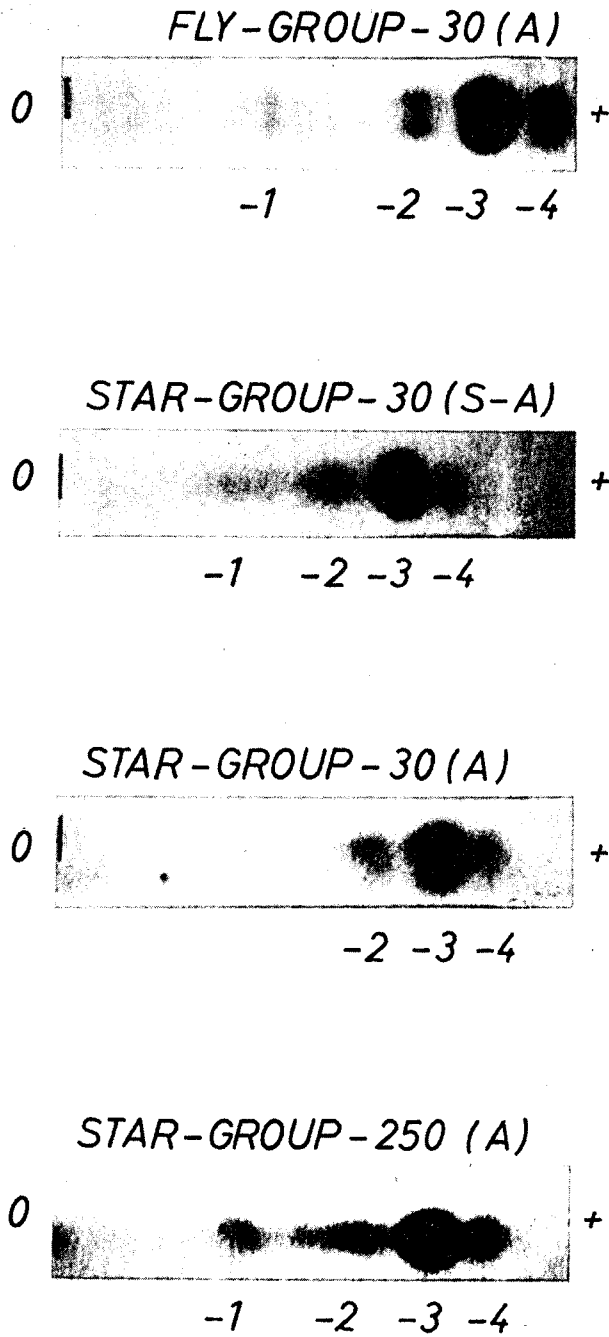


Fig. 5. Anodic side of electrophoretic maps corresponding to those of Fig. 1. + =: anodic end. Other symbols as in Fig. 4.

untrained mantids differed in levels of activity, motivation and attention.

The second series of experiments included two changes as compared with the first series. First, ascending chromatography was omitted, since results from the former series had demonstrated that maps from trained and untrained mantids differed only in the horizontal dimension. Second, trained mantids were taken not only from the star-group 250 but also from the star-group 30 and the fly-group 30. The results of this series of experiments are clear-cut.

(1) Electrophoretic maps corresponding to mantids that showed a significant decrement in number of attacks during training, i.e. attackers and sub-attackers of the star-group 250 and attackers of the star-group 30, revealed two extra ninhydrin positive components that were not present in any map of the control-group. It is significant that the attackers of the star-group 30 received a session  $e_1$  of only 20 trials and that all the training totalled only 30 trials. On the other hand, the sub-attackers of the star-group 30, unlike those of the star-group 250, did not show any significant decrement in the number of attacks during  $e_2$  nor any extra-components in their maps.

(2) Mantids of the fly-group were shown a stimulus that proved to be more attractive than the rotating star and no decrement in their performance was observed during training, i.e. the total number of strikes during  $e_1 + e_2$  by the attackers of the fly-group 30 was markedly greater than that of the star-group 30. Therefore, it is valid to assume that levels of activity, motivation, attention and punishment were higher during the training of the fly-group 30 than during the training of the star-group 30. However, electrophoretic maps from brains of the attackers of the fly-group 30 turned out to be similar to those of the control-group.

(3) This set of findings leads us to conclude that the appearance of the amine-containing components +2 and +3 in the electrophoretic maps is correlated only with decrement in the attack performance during training. Since MALDONADO (1972) demonstrated that this decrement fulfils the parametric conditions of a learning process, it is difficult to escape the conclusion that a chemical correlate of learning has been found. However, this does not imply a casual relationship between these variables. The precise role of these 'chemicals' in the learning process and the possibility that one memory could be identified with one discrete molecular trace (HYDÉN and EGYHAZI, 1962) are still the subject of investigation.

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