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Change of Protein-synthetic Patterns with Habituations of Amoeba and Planaria by the Light Stimuli

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光에 대한 Amoeba와 Planaria의 습성화에 따른 蛋白質 合成類型의 變化

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ABSTRACT

When the light stimulus was presented continuously, habituations were formed, after 13 and 14 hrs in amoeba and planaria, repectively. As amoeba more habituated, the light-escaping velocity of streaming was decreased with a negative-exponential function.

In two-dimensional electrophoretic patterns of proteins of amoeba habituated, a newly synthesized protein (pI 7.0, MW 20 kD) was detected.

The ratio of light-escape of planaria was also decreased with a negative-exponential function. In the protein patterns of these habituated planaria, two newly svsnthesized proteins (pI 7.5, MW 20 kD, and pI 7.7, MW 80 kD) were detected.

Therefore, it is conjectured that similarity or commonality may exist between amoeba and planaria habituations to light, and in these behavioral modifications, specific protein(s) may be involved in molecular level.

INTRODUCTION

Habituation is defined traditionally as a "waning of a response as a result of repeated stimulation" (Thompson and Spender, 1966). If a stimulus that typically elicits an innate reponse is presented repeatedly, the reponse may weaken or even disappear. However, it is not due to the fatigue of a cell or tissue damage, but is a stimulus-specific and relatively enduring phenomenon (Applewhite, 1973; Wood, 1973; Barnett, 1981). Therefore, habituation may be considered as a simple form of learning (aquisition of a reponse).

Recently, many studies showed that proteins are involved in the behavioral modifications such as circadian rhythm, phototropism, chemotaxis, neuronal and endocrine functions (Springer *et al.*, 1979; Pollock *et al.*, 1983; Yeung and Eskin, 1987).

The amoeba and the planaria are useful in a test for the habituation to light stimulus, because they show a negative phototaxis. Several stimuli will halt amoeboid movement and locomotion.

Certain stimuli (e.g. light), however, even if continued at the shock level, are eventually tolerated, and the amoeba adapts or habituates and resumes its locomotion (Jeon, 1973). Analysis of time relationship between the phases of activities of pseudopodia in freely moving polytactic amoeba demonstrated that they are correlated (Klopocka and Grebecki, 1980). Stimulation by light promises to be particularly convenient in behavior study of amoeba, because it is easy to control its intensity in study of amoeba, because it is easy to control its intensity localization and time action. Recently, localized photostimuli have been applied to amoeba in the form of bright and dark areas projected upon the plane of migration of amoeba (Grebecki, 1981).

Westerman (1963) reported that there are at least two types of habituation to light in planaria a relatively temporary effect that showed up as a decrement in responding within a given day's trial and a relatively lasting effect that appeared as a response decrement across several days of testing. Analysis of nucleic acids from planaria removed at various stage of conditioning showed that the RNA/DNA specific activity ratios were siginificantly different in trained animal during the middle stage of training (Corning and Freed, 1968).

It is, therefore, necessary to charaterize the biochemical properties of the habituation of ameoba and planaria, to elucidate the basic phenomenon of learing.

This is attempted to examine the similarity of habituation patterns and its biochemical aspects which underlie these processes.

MATERIALS AND METHODS

Culture of Amoeba (Amoeba sp.)

Isolation and culture of food organisms; *Tetrahymena* sp.

Amoeba and *Tetrahymena* were originally collected in Kong-Ju, Korea by professor Park Young-Cheol in 1985.

Tetrahymena were isolated with micromodulator in the sterile chamber under the disecting microscope, and washed more than five times in the sterilized glass-distilled water then innoculated to the axenic culture medium (Ahn, 1983), containing 0.1% yeast extract and 2% proteose peptone. *Tetrahymena* sp. were axenically grown in constant temperature shaker at 20°C.

Culture medium

Modified Chalkley's solution. (Jeon and Jeon, 1975) was used in both stock and mass culture of amoeba. Stock solution for the culture media was prepared as follows, solution A contained of 0.25 mole NaCl, 0.025 mole KCl, 0.025 mole $NaHCO_3$, 0.004 mole Na_2HPO_4. H_2O in 500 ml of distilled water. Solution B consisted of 0.05 mole $CaCl_2$. $2H_2O$ in 250 ml of distilled water. Working solution was prepared by adding 2 ml of solution A and 1 ml of solution B to 1,000 ml ofdistilled water.

Stock culture

Stock culture of amoeba was kept as described by Lorch and Danielli (1953).

Amoebae were cultured in completely dark at 20 ± 2°C in petridishes (18 cm in diameter) with *Tetrahymena* as food organisms. The culture were renewed once a week in clean dishes with about 1,000 amoebae in each. The renewed dishes were supplied with *Tetrahymena* and

about 10 boiled wheat grains, where the latter were needed for maintenance of molds and bacteria which in turn served as food for *Tetrahymena*.

Mass culture

Amoebae were harvested from the stock culture, and mass-cultured to obtain a large population.

In mass culture, amoebae were grown with *Tetrahymena* as food organisms in stainless dish (25 cm × 30 cm) in complete darkness at 20±2°C. All dishes were covered with black acrylplates. Amoebae were fed three or four times a week with fresh Chalkley's solution depending on the need of amoebae for the experiments. All feeding an exchanging of Chalkley's solution were performed in the semidark room.

Cell line culture

Monoclonal population of amoeba were obtained by single cell culture. Healthy amoeba were picked up from mass culture and placed single in syracuse watch glasses. The amoeba in single cell culture was washed and fed with *Tetrahymena* every other day. The amoebae of cell line culture were used in experiments.

Collection and Culture of Planaria (*Dugesia* sp.)

Planaria were collected at stream of Mt. Kwan-ak in Seoul using the pieces of beef liver. The collecting jars were wrapped with alluminum foil, and brought into the laboratory.

Upon arrival at the laboratory and planarians varying in length from 8 to 20 mm, were housed in large opaque container filled with balanced aquarium water. The planarians were kept in a light-proof temperature-controlled (at 18±2°C) environment, and were fed chopped raw beef liver, two or three times a week. Aeration of water was carried out for about half an hour everyday. They were used for experiments two days after the last feeding.

Induction of Habituation and Its Measurements

Amoeba

Amoeba were used for experiments 2 days after the last feeding, when they manifest the highest locomotive activity.

Amoeba were placed in a culture dishes, and illuminated with a standard incandescent lamp with the intensity of 8000 lux, and the temperature was constantly maintained at 20±2°C as shown in Fig. 1.

At the intervals of 1 hr, healthy individuals were transferred to a slide, the half side of which was screened by black tape and aluminum foil, together with some drops of the original culture medium. The slides produced in that way were introduced on the stage of the microscope ready for the experiment and left for 10-15 min, before the beginning of the observation; this allowed the amoeba to adapt to the new condition.

The light intensity amounted to 8,000 lux in the bright part of all field of view, and was reduced approximately by the factor of 4 within the screened shade. The overall time consumption for the complete movement of body from bright part to screened dark area was measured at 1 hr-intervals. The records were taken in a semi-dark room at 20±2°C, and from these data, then the habituation curve was later plotted.

Planarians

Like amoebae, planarians were used for experiment 2 days after the last feeding.

Planarians kept in dark environment were transferred in a stainless steel dish (15 cm × 20 cm), and the light intensity illuminated was 12,000 lux, which is white light produced by a standard incandescent lamp. Temperature was maintained at 18±2°C with tap water at all times. Fig. 2. shows the design of the apparatus which was used for the presentation of stimuli to large numbers of planarians.

At intervals of 1 hr, the ratios of individuals which migrated into the shaded area made by aluminum foil at each side (4 cm in width) and still remained in bright one was measured after 30 sec of shade were measured. These data were plotted to form a habituation curve.

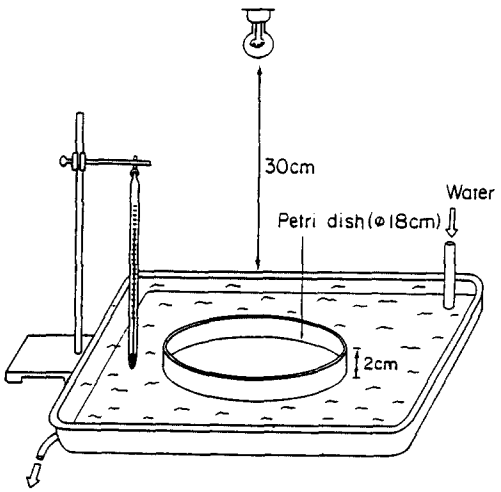


Fig. 1. The design of the apparatus which was used for the presentation of stimulus to large population of amoebae. Intensity of light stimulus was 8,000 lux, and the temperature was maintained at 20±2°C by constant flowing of tap water. At intervals of 1 hr, the velocity of streaming was measured on half-screened by black tape and aluminum foil in semidark room.

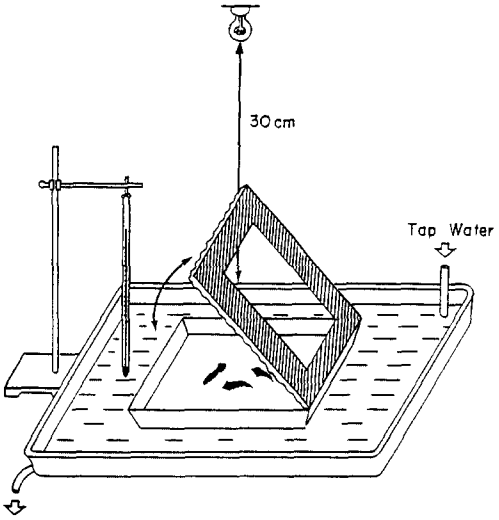


Fig. 2. Light-stimulating apparatus for large number of planarians (150 individuals). Light intensity was 12,000 lux, and temperature was controled constantly at 18±2°C by flowing the tap water. At intervals of 1 hr, the ratio of responsive and nonresponsive planaians by shading the 4-sides with aluminum foil was determined as measure of habituation.

Preparation of Protein Samples and Assay

Amoeba

Before harvest, amoebae were starved for 3 days to remove the proteins originating from food organisms.

They were washed 3 times with Chalkley's solution and transferred to 45 ml centrifuge tube, twice more by centrifugation for 2 min, at 200 *g*. To the cell pellet equal volume of 0.02M TH buffer (pH 7.4) was added and transferred into a glass homogenizer in an ice box. The homogenate was centrifuged (Beckman Model J-21 B) for 15 min at 10,000 *g*. The supernatant contining water-soluble protein was collected and kept at −20° for future use.

Planaria

Planarians were harvested 3 days after last feeding to eliminate the proteins originating from food materials.

They were also washed 3 times with 0.02 M TH buffer pH 7.4 and transferred into a glass homogenizer in an ice box. Homogenation was performed in 5 ml of a mixture containing 0.1% Triton X-100 and 0.13% sodium deoxycholate, solution A (25 mM sucrose, 5 mM Hepes, pH 7.4, 25 mM KCl, 0.5 mM $MgCl_2$) and 0.2% sodium dodesyl sulfate (Retz and Steele, 1977). The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant except the upper lipid layer was collected with a syringe and added 30% (w/v) trichloroacetic acid (TCA) until the final concentration to 5%. The samples were mixed on a vortex mixer and placed in an ice bath, for 15 min and then centrifuged for 5 min at 4,500 *g* (Sorval GL C-2B) to pellet the protein. In this step the brown pigments of planarians were removed. The pellets were suspended in glass-distilled water by homogenation and precipitated by centrifugation as before. This step was repeated twice to remove excess TCA. The pellets were dissolved in lysis buffer containing 9.5 M urea, 2% (w/v) NP-40, 2% ampholines (comprised of 1.6% pH range 5 to 8, 0.4% pH range 3 to 10) and 5% β-mercaptoethanol. The lysate was centrifuged for 15 min at 10,000 *g* to remove undissolved materials and supernatant was collected, then immediately used in protein assay and electrophoresis.

Protein assay

Protein concentration in each sample was determined according to Lowry *et al.* (1951) using bovine serum albumine (BSA) as a standard. The absorbance of each sample was measured in Spectronic-20 at a wavelength of 750 nm. When the sample were treated with Triton X-100, modified Lowry assay was used (Sottocassa, 1971).

Electrophoresis

Isoelectric focusing

minor modification. IEF gel was made in glass tubes (13×20 mm, inner diameter) sealed at the buttom with parafilm. The gel mixture contained 9.0 M urea, 4% (w/v) acrylamide, 2% (v/v) NP-40, 2% ampholine (1.6% pH 5-8 and 0.4% 3.5-10) in final concentration.

One volume of protein sample was mixed with another volume of lysis buffer. In each gel, 400 μg of protein was applied. The upper and bottom reservior was filled with 0.02 M NaOH (anode solution) and 0.01 M phosphoric acid (cathode solution).

IEF gels were run at 200 V for 20 min, 300 V for 30 min, 400 V for for 30 min, and 600 V for 14 hrs followed by 800 V 1 hr. After IEF, the gels were removed by pressure on 30 ml syringe which was connected to the tube. The gel were soaked individually in the 5 ml treatment buffer and equilibrated for 1 hr.

SDS-PAGE

The second dimension was performed essentially according to the method of Laemmli (1972). Separating gel was made of 8-14% gradient gel containing 0.132 M TH-buffer pH 8.8 and 0.1% SDS. Stacking gel consisted of 3% acrylamide, 0.125 M TH buffer pH 6.8, and 0.1% SDS was overlaid to the separating gel. Ammonium persulfate and N,N,N′, N′-tetramethylene diamide (TEMED) were used to catalize the polymerization of the gel.

Gel staining

After the completion of electrophoresis, gels were fixed and stained for 6-8 hrs in a solution containing 0.125% coommassie brilliant blue R, 50% methanol, and 10% acetic acid. Gels were destained 7-8 hrs in 50% methanol and 10% acetic acid, then stored in 10% methanol and 5% acetic acid.

To enhance staining sensitivity silver staining method of Merril (1981) was applied. In silver staining, water purity and staining temperature were crucial for generating contrast (Nielson and Brown, 1984). Particulary silver nitrate solution prepared with non-deionized was silky nitrate solution prepared with non-deionized was silky and made poor resolution.

RESULTS

The Behavior of Planaria to Light

Typically, there are two forms of amoeba: one is monotactic or monopodial, orthotactic with only one single front and the other polytactic or polypodial with two or more pseudopodia as shown in Fig. 3.

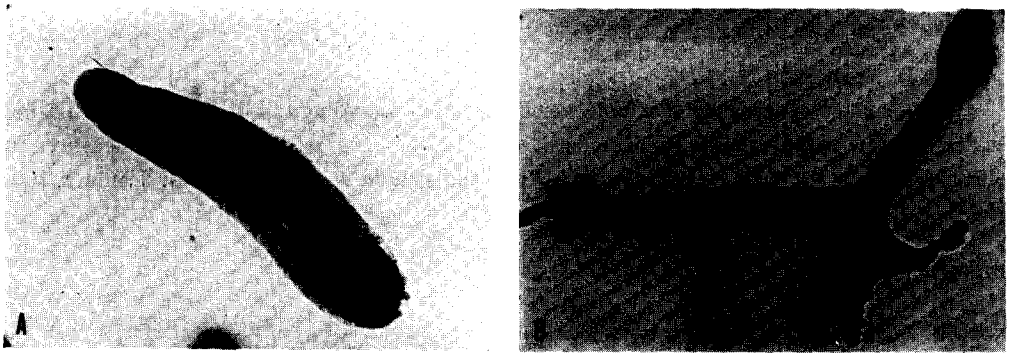


Fig. 3. Tow forms of amoebae in free-living states.
(A) Monotactic (or monopodial, orthotactic) form with only one single front
(B) Polytactic (or polypodial) form with two or more pseudopodia (×500).

Reactions of different pseudopodia of amoeba observed after shading the tip of one of them, that is, the general photonegative responses presented in Fig. 4. Amoeba cultured in dark environment migrate into the dark-screened area at the average rate of streaming of 4,920 μm/sec. The serial pictures showed the successive phases of responses manifested by the shaded pseudopodium. The gradual changing pattern of extention and retraction of different fronts of amoeba (as indicated by arrows) strongly suggests that the withdrawal of pseudopodia that remain in bright area is delayed; it begins when the extention of shaded pseudopodium is already well advanced. A more detailed study of this phenomenon was undertaken by means of frame-by-frame analysis of cinematographic records by Gebecki and Klopocka (1981).

At beginning of the experiment the cells are polytactic. Formation of advancing pseudopodia at the first tage was shown in Fig. 4. At the same time, the velocity of urodial retraction is slightly increased and the other pseudopodia at the bright area are retracted. Then the advancing pseudopodia was gradually increased. During the first and second stages, when amoeba has two

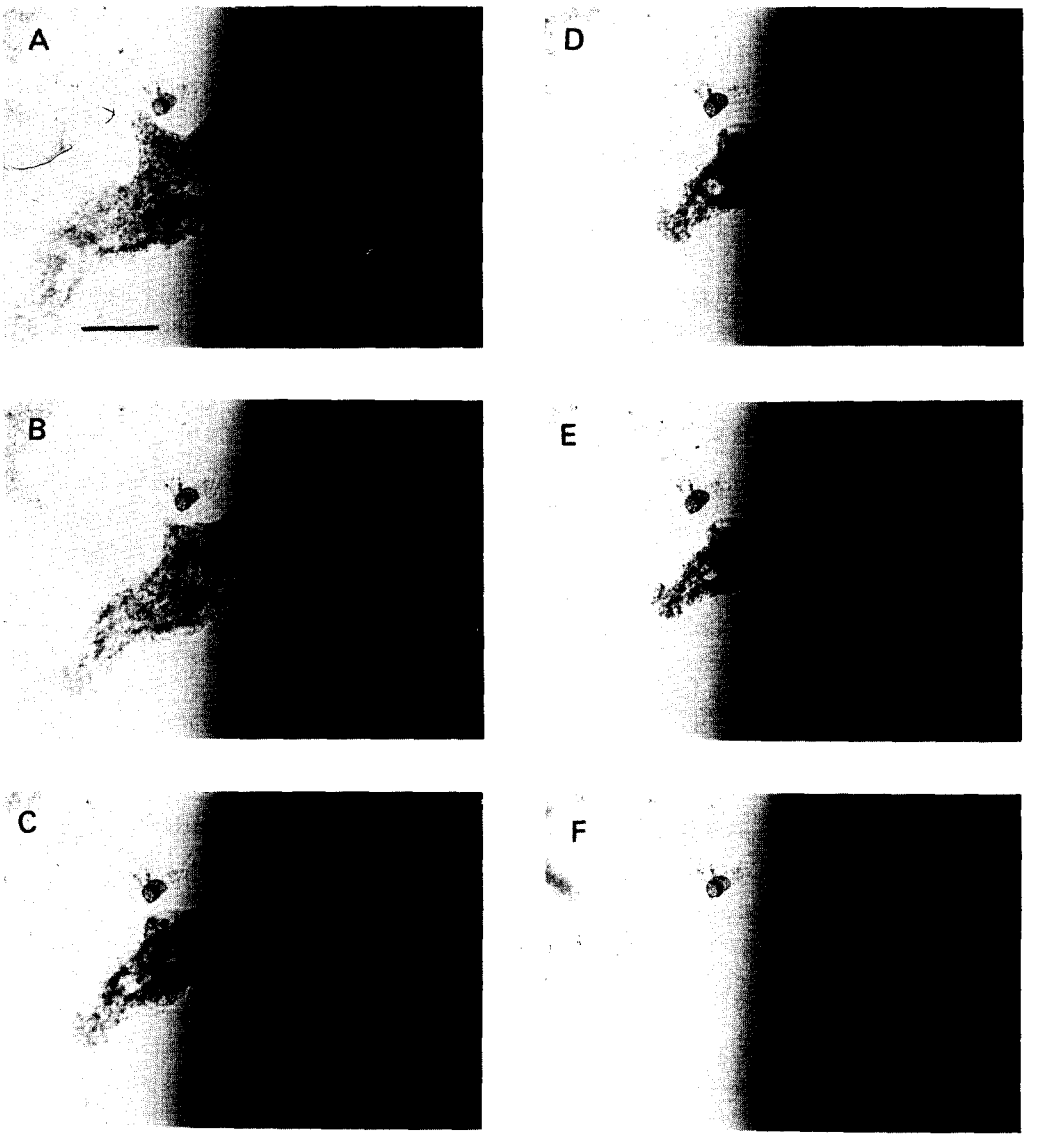


Fig. 4. Behavior of a amoeba obliged to follow the half-screened shade of slide after a period (15 min) of free migration (×500).

(A) The pseudopodium which contact to shade was activated before other psudopodia began to contract.

(B) The shade contacting front advance vigorously while others retract.

(C-E) The overall body gradually migrate into the dark side.

(F) Amoeba moved into shade completely (after about 135 sec).

The average velocity of amoeba grown in complete dark was 4,920 μm/sec. Bar indicates 100 μm.

active fronts, the progression of the prospective advancing pseudopodium is from begining faster than that of the future contracting pseudopodium.

Three successive phases may be distinguished in the course of each experiment. The first one represents the period of first locomotion of an affected amoeba before stimulation. The second one corresponds to the lapse of time (5-15 sec) between the application of shade and the beginning of the visible reaction of the shaded pseudopodium. In the third phase the pseudopodium exposed to the lower level of illuminosity reacts vigorously and the effects become manifest in others.

The functional interdependance of pseudopodia in *Amoeba proteus* stimulated by light-shade difference was discribed in detail by Grebecki and Klopocka (1981), and Klopocka and Grebecki (1982).

The Behavior of Planaria to Light

When planarians were placed in a field of light at the intensity of 12,000 lux (about 110 foot-candle) or more they shows wigwag responses. If one side of container or their environment were dark or lower illumination, planarians orient themselves progressively faster to that side, that is, they represent the photonegative responses.

If a number of specimen of planarians are moving in a certain direction, the direction of the light is changed so as to fall upon their anterior ends, they usually turn the head from side to side two or three times, then follow up these movements by turning the body till it is finally directed away from light, this behavior of planaria was called" testing movements" by Jennings (1976).

Behavioral Changes in Amoeba and Planaria as a Result of Habituation to Light

In the study of habituation to the light stimulus in the protozoan amoeba and flatworm planaria, following results were obtained.

These protozoa and flatworm showed fast photonegative movement toward dark or shade in response to initial presentations of stimulus, but eventually less responsive to light as it continued.

The experiments were concerned in both behavioral and biochemical aspects of habituation.

Fig. 5 shows that the velocity of streaming of amoeba migrating toward the screened dark side decreases as a function of time over a period of 13 hrs. That is, as they became more habituated to the light stimulus, the rate of locomotion is decreased, and the velocity decrement was used as the mesure of habituation. A non-zero asymptotic level of velocity which is regarded as habituation is then attained. This velocity decrement was approximately a negative exponential function of the time expressed to light ($r = -0.996$).

This habituation patterns of amoebae's to light shock were similar to that reported previously in protozoan, *Stentor coerlius* (Wood, 1973). An indication that the motile mechanism was not fatigued was given by the fact that an electrical stimulus (5-7 mA) produced streaming toward the cathode and it began at the tip of forming, exactly as in spontaneous pseudopodium formation, if the stimulus was given immediately after the habituation.

In planaria, light stimulus was presented at the intensity of 12,000 lux (about 110-foot-candle), and the ratios of individuals which migrated into the shaded area and still remained in light one

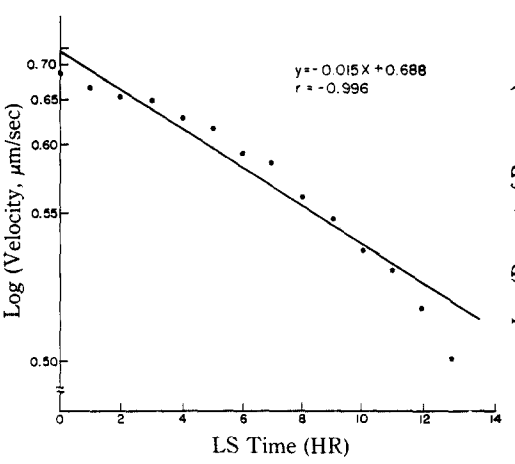


Fig. 5. Logarithmic plot of the velocity of streaming in amoeba less the asymptotic level of response showing that the observed velocity decrement curve is approximated by a negative exponential function of the duration of light stimulus.

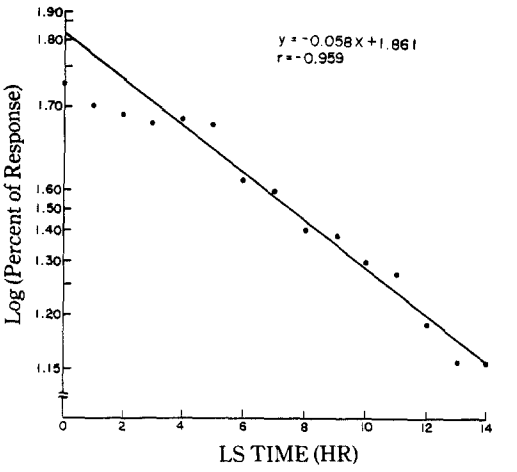


Fig. 6. Logarithmic plot of the response ratios in planarians (*Dugesia* sp.) less the asymptotic level of response showing that the observed decrement of the ratio is approximated by a negative exponential function of the light-stimulus time.

were used as measures of habituation. The results were shown in Fig. 6, at first the ratio was 0.562, that is, 56.2% of 150 planarians migrate to the shaded area. As they became more habituated to light stimulus, the ratio was decreased to 8.22%, which was asymptotic level regarded as the point of habituation formation (14 hrs).

This habituation pattern was simillar to that of amoeba, except the some initial period (1-3 hrs) and the time required for the formation of habituation, which 13 hrs in amoeba and 14 hrs in planaria. The response decrement was also approximately a negative exponential function of the time exposed to light (Fig. 6, $r = -0.959$).

These planarians habituated to light, too, reponded to other stimuli, e.g. mechanical or electrical stimuli. This indicates that the motile mechanism was not fatigued, consequently, the behavioral analysis presented above shows that the response decrement noted in amoeba is analogous to the phenomenon of habituation noted in planaria.

Change of Synthetic Patterns of Proteins as a Result of Habituation to LIght

The two-dimensional electrophoretic patterns of water soluble proteins of amoeba are shown in Fig. 7. Fig. 7A represents the protein pattern of dark cultured control group, Fig. 7B shows that of the habituated to light for 13 hrs. In habituated amoeba, a protein with pI 7.0 and molecular weight 20,000 daltons was newly synthesized.

The protein composition of planarians grown in dark and habituated to light for 14 hrs was also compared by two-dimensional SDS-PAGE. In habituated planarians, there were two new protein spots appeared in the gel (Fig. 8). These have pI 7.5 , pI 7.7, and molecular weight 20,000 and 80,000 daltons, respectively.

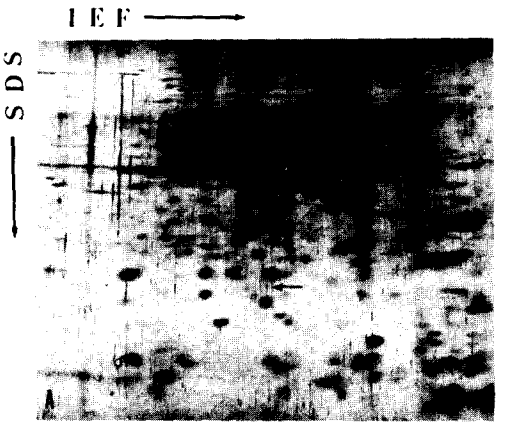
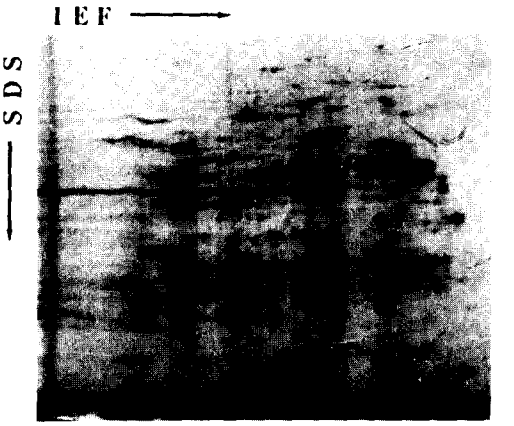


Fig. 7. Two-dimensional electrophoretic patterns of amoeba. The gels were stained with 0.125% coomassie brilliant blue, and stained again with 0.12 M silver nitrate.
(A) Water soluble protein of dark-grown amoeba (control) at 20±2°C.
(B) Protein pattern of light-habituated amoeba at 20±2°C. A newly synthesized protein pI 7.0 and MW 20,000 daltons was indicated by an arrow.



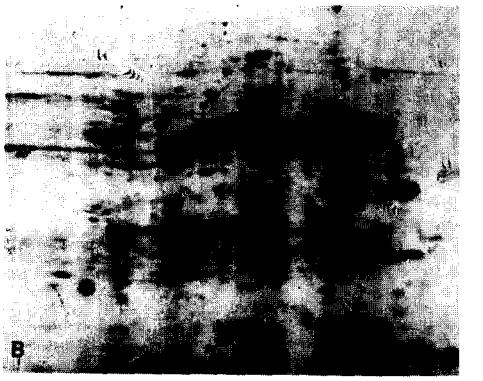


Fig. 8. Protein patterns of planaria.
The gels were stained with 0.125% coomassie brilliant blue, and stained again with 0.012 M silver nitrate.
(A) Electrophoretic pattern of completely dark-grown planaria at 18±2°C.
(B) Proteins of light-habituated planaria at 18±2°C.
Two newly synthesized proteins (pI 7.5, MW 20,000 and pI 7.7 MW 80,000 daltons) were indicated by arrows.

Newly synthesized protein of 20,000 daltons as a consequence of habituation was common in both of amoeba and planaria, although the pIs were slightly different.

This is suggestive of some similarity or commonality in biochemical aspects of habituation between amoeba and planaria.

DISCUSSION

Conclusions as to the biochemical basis of habituation in protozoan amoeba and flatworm planaria can be made on behavioral evidence alone.

In order to make such an analysis a simple models were proposed for the events which occur in individual amoeba and planaria between the time of the stimuli and the responses.

The results of this study show the pattern of habituation to light in amoeba and planaria is similar each other. In other words, amoeba and planarian habituations were characterized by a similar response decrement which is approximated by a negative exponential function of the light-stimulated time (Fig. 5, Fig. 6). A little differences in the rate of habituation between them were noted that the slope of negative exponential function was 0.015 in amoeba and -0.058. in planaria

Consequently the term habituation will be used to describe these amoeba and planarian behavioral changes and it is to understand that the use of a common term to describe the behavior of protozoa and flatworm implies that some commonality can be also found in the physiological and other biochemical changes which underlie these phenomena. Several reviews of data on habituation have consistently noted the similarity between metazoan and the response decrements noted in protozoa (Jacobson, 1963; Wood, 1973).

Therefore, it can be supposed that the electrophysiological and biochemical bases of these two processes might also be similar.

In this event amoeba and planaria may come to serve as model systems for the elucidation of simple learning phenomena. Levison and Gavurin (1979) reported that planaria habituated to the light shock increased, but they did not analyze the changes of protein-synthetic patterns. In the study of learning behavior of planaria, Chang (1983) and Chang *et al.* (1983) showed that as the planaria became more habituated to light, their light-escaping velocity was decreased, and as the days of dark treatment increased the velocity was increased. However, there was no protein change in one-dimensional electrophoretic patterns between the light-habituated and the dark-treated planarians. And several researchs were concenred in the involvement of proteins in behavior modifications. In the prokaryotes and eukaryotes methyl groups could be added to and removed from the carboxyl groups of proteins and these reactions have a role in several behavioral chemotaxis, leucocyte chemotaxis, neural and endocrine functions (Springer *et al.*, 1979). Pollock *et al.* (1983) have reported that 60,000 daltons plasmamembrane flavo-protein were involved in phototropism of the fungus *Phycomyces.* Recently, Yeung and Eskin (1987) demonstrated that a specific protein (34,000 daltons) was involved in the regulation of a circadian rhythm in *Aplysia* eye. In addition, Ungar *et al.* (1972) isolated a specific peptide from dark-avoidence trained rats by gel filtration on Sephadex and thin-layer chromatography, then called this peptide scotophobin. The peptides associated with sound habituation were also extracted and tested for habituation-inducing activity then named ameletin. Although the exact structure of the substance formed in the brain of green avoiding and blue preferring fish still remains to be specified, the two peptides, from blue-avoiding or green-avoiding animals, share a common sequence of seven or nine amino acids. (Tate *et al.* 1976). With these experiment data, Ungai (1980) suggested "one peptide, one behavior" hypotehsis that a different brain peptide corresponds to each learned behavior.

In this study of habituations in amoeba and planaria, as a result of their habituation to light stimuli, three newly synthesized proteins were detected in the gels; one protein (pI 7.0, molecular

weight 20,000 daltons) in amoeba, two (pI 7.5, pI 7.7, molecular weight 20,000 and 80,000 daltons, respectively) in planaria.

It may be conjectured that these proteins would be involed habituations to stimuli, of course, including light. In addition to, it is noticeable that the common proteins (20,000 daltons) of amoeba and planaria, even though the isoelectric points were different slightly, may be crucial to habituations. But it is possible that both of two proteins in planaria may be involved together or protein of 80,000 daltons more crucial than 20,000 daltons, or may have a role in sequential manner, i.e., that of 20,000 daltons in planaria may be the result of primary habituation, then it regulates or affects the secondary habituation or vice versa.

No matter how the mechanisms are, it seems that protein(s) may be involved in behavioral modifications, including habituation, of amoeba and planaria.

摘 要

광자극을 연속적으로 주었을 때, amoeba는 13시간 후에, planaria는 14시간 후에 습성화가 이루어졌다.

amoeba의 습성화가 진행됨에 따라, 광회피 속도는 음지수 함수적으로 감소하였다. 습성화된 amoeba의 2차원 전기영동법에 의한 단백질 분석결과 새로운 단백질(pl 7.0, 분자량 200 kD)이 발견되었다.

planria의 광회피 비율도 음지수 함수적으로 감소하였다. 습성화가 이루어진 planaria의 단백질분석 결과 두가지 단백질(pl 7.5, 분자량 20 kD)이 새로 합성되었다.

그러므로, amoeba와 planaria의 습성화 양상은 유사하며, 이러한 행동변화에는 분자적인 수준에서 특정한 단백질이 관여한다고 사료된다.

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