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EFFECT OF SEROTONIN AND OTHER BIOACTIVE AGENTS ON THE RHYTHMIC ACTIVITY IN THE GLOCHIDIA OF FRESH-WATER MUSSEL (*ANODONTA CYGNEA* L.)

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Abstract—1. The effect of different, biologically active agents (serotonin, L-tryptophan, 5 OH-tryptophan, tryptamin, adrenalin, noradrenalin, tryamine, and drugs (LSD, chlorpromazin, iproniazid) was investigated on opening and closing of the glochidia of *Anodonta cygnea* L. We found that:

a. All the employed bioactive agents have a more or less intensive activity-increasing effect.

b. When using tryptamin, the increase of activity was long-lasting, keeping on for over 30 min.

c. LSD and to a smaller degree chlorpromazin cause an increase of activity. These agents and iproniazid inhibit the effect of tryptamin.

2. It could be suggested that in the early ontogenetic stage of *Anodonta*, tryptamin, formed during the course of metabolism, plays the role of a local hormone in the regulation of the "spontaneous" rhythmic activity.

INTRODUCTION

THE result of our previous investigations (Lábos & Salánki, 1963) on the parasite larvae (glochidia) of the fresh-water mussel showed that their rhythmic activity can be increased by different inorganic salts. An especially important role is attributed from this point of view to potassium chloride which in a concentration of $1–2 \times 10^{-3}$ M results in a long-lasting increased activity. As this effect is probably due to the well-known direct depolarizing effect of potassium, nothing can be concluded from it concerning the specific basis of the rhythmic activity in glochidia. For this reason we wanted to find what kind of the bioactive agents might have an influence in this early ontogenetic state of mussels on the rhythmic activity. From the results we expected to learn more about the agent or agents involved under physiological conditions in the regulation of the rhythmic activity in glochidia. The obtained data might give certain information about the ontogenesis of the neurohumoral regulation as well as a more satisfactory understanding of the mechanism of rhythmic activity.

In the embryos of the marine gastropods Koshtoyants, Buznikov & Manukhin (1961) demonstrated that the beat of the cilia can be significantly increased by adding low concentrations of serotonin. Numerous other data (Erspamer &

Ghiretti, 1951; Welsh, 1957, 1958; Koshtoyants, 1957; Hill, 1958; Koshtoyants & Rózsa, 1961a; Salánki, 1963) also refer to the important role of serotonin in molluscs.

In adult molluscs other biologically active agents must be taken in consideration. These are acetylcholine (Tauc & Gerschenfeld, 1961; Fänge & Fuggeli, 1962), adrenalin and noradrenalin (Euler, 1961; Koshtoyants & Rózsa, 1961b). Their importance, however, could not be proved from Manukhin's and Buznikov's experiments (1961) in the early stage of ontogenesis in gastropods. In this paper we give an account of the results obtained by employing serotonin, its related compounds, catecholamines and pharmacons.

METHODS

The glochidia were obtained from the external gills of the adult animals where they develop and stay from autumn till early spring in great number. This organ is their temporary living-space from the time of their formation until their release into the open water. As the glochidia are covered with a mucous material first they were rinsed thoroughly in tap water, then grouped with a micropipette into twenty-five individuals. We investigated the activity of the group in counting the number of quick closures of the individual glochidia in every minute. The number of contractions per minute was observed in at least four groups so the values "activity/min" in the figures are always given for 100 glochidia. The investigations (the larvae are about $250 \times 400\ \mu$) were carried out under binocular microscope with direct observation.

The motor activity is connected with the action of the embryonic adductor muscle and consists of the closure and opening of the larval shell valves. Before adding the agents to be investigated, control counting took place. This "background" or "spontaneous" activity gave—calculated in general for 100 glochidia—a value under 20/min. In some of the groups no spontaneous activity could be detected during the control time.

The agents in question were added to the glochidia after the tap water was drained; the observations were continued for 30–60 min depending on the results and noting the number of the contractions in every minute. The agents were applied in concentrations of 0·01–100 μg/ml, and to the groups (each containing as already mentioned twenty-five glochidia) 0·25 ml of solution was given. As the control was carried out in tap water the agents were dissolved in tap water.

RESULTS

Effect of serotonin and related compounds

Within this group the effect of serotonin, 5 OH-tryptophan, L-tryptophan and tryptamin was investigated. The results are shown in Figs. 1 and 2. The ordinate gives the number of closures of 100 glochidia/min. On the abscissa the time is given in minutes, to the left the control values, to the right the time after the investigated agent was added. All effective concentrations of all employed chemicals were evaluated and demonstrated as follows:

(a) serotonin 0·1–1–10–100 μg/ml;
(b) 5 OH-tryptophan 1–10–100 μg/ml;
(c) L-tryptophan 1–10–100 μg/ml;
(d) tryptamin 0·01–0·1–1–10–100 μg/ml.

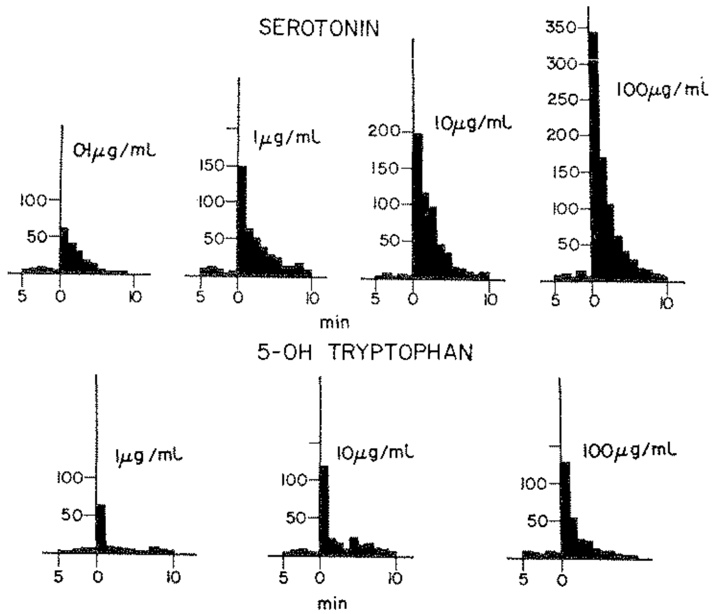

FIG. 1. Effect of serotonin and 5 hydroxytryptophan on the rhythmic activity of the glochidia. Abscissa, time in minutes; Ordinate, number of contractions per minute.

Comparing the results it was observable that the most significant initial increase of activity was caused by serotonin in 100 μg/ml concentration and that its effect ceased by the end of the tenth minute. A similar decrease in activity takes place in cases of smaller concentrations of serotonin. The activity-increasing effect can not be produced again by repeated doses of serotonin.

When using 5 OH-tryptophan and L-tryptophan in 10 and 1 μg/ml concentrations we observed an increase of activity similar to that due to serotonin during the first minute, but the cessation of the effect was very quick and took place within 2–3 min.

In the case of tryptamin even a concentration of 0·01 μg/ml caused a definite increase of activity. However, the initial value of the increasing activity was in all cases smaller (remaining under 100/min) than when adding other tryptophan derivates. At the same time the length of the effect differs significantly from the other. We found, namely, that tryptamin causes a lasting increase in activity.

This effect is clearly seen at 1 μg/ml concentration, but it is even more clear when adding 10 and 100 μg/ml concentrations of tryptamin. The effect was seen over the 10 min period of examination.

In single cases we found high tryptamin sensitivity when addition of 100 μg/ml tryptamin caused a long-lasting and very high increase of activity. Closing of the glochidia also took place due to the continued contraction of the embryonal

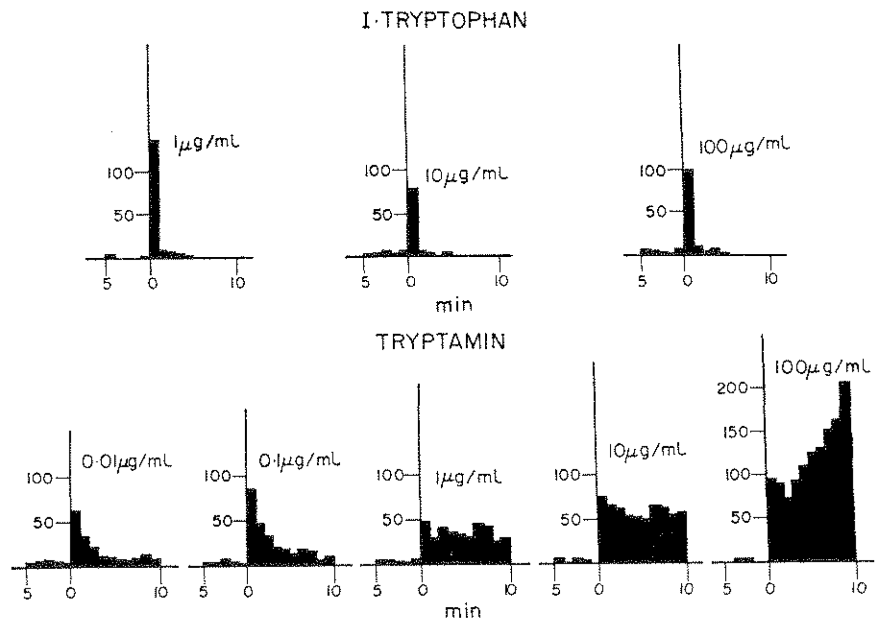

Fig. 2. Effect of L-tryptophan and tryptamin on the rhythmic activity of the glochidia. Abscissa, time in minutes; Ordinate, number of contractions per minute.

adductor muscle. In Fig. 3 a similar case is demonstrated, where by the end of the twentieth minute 80 per cent of the glochidia were in a closed state. A similar lasting increase of activity was not detectable from serotonin, 5 OH-tryptophan or L-tryptophan, as their effect ended within 3–10 min and the glochidia did not close even at greater concentration.

With regard to the data (Udenfriend *et al.*, 1956) stating that serotonin is synthesized in the organism from L-tryptophan via 5 OH-tryptophan, we examined the effect of the latter substances throughout 30–60 min in order to see whether the increase of activity takes place or not during this period. These investigations, however, gave negative results.

In February and in March, when the glochidia are liberated into open water, significant results were obtained with tryptamin. In this period the sensitivity to tryptamin subsides extraordinarily compared to earlier periods and using even a

100 μg/ml concentration we could not observe higher values than 20–30 contractions/min in the first minutes. Continued observation showed that the increase of activity becomes rapid and, as it is seen in Fig. 4, by the twentieth minute it reaches 180/min. A similar effect could not be detected in the case of the other tryptophan derivates.

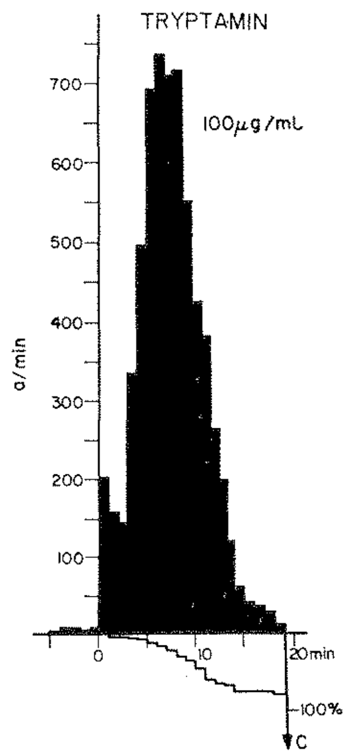

FIG. 3. Effect of tryptamin at 100 μg/ml on glochidial activity during autumn. The lower curve shows the percentage of glochidia that are closed.

The effect of catecholamines

Among catecholamines the effects of adrenalin, noradrenalin and tyramin were investigated in 0·01, 0·1, 1 and 10 μg/ml concentrations. We found that most the effective concentrations of all the three compounds was the 1 μg/ml, producing an activity of about 100/min (Figs. 5–7). In the case of adrenalin this proved to be the only effective concentration; under or above it no effect could be detected. For noradrenalin or tryptamin, a decrease of activity took place at concentrations over 1 μg/ml and disappeared completely at a concentration of 10 μg/ml.

Investigating the duration of the effect we found that at all concentrations of catecholamines the increase of activity was of short duration and ceased in all cases within 5 min.

However, it should be mentioned that after the fifth minute not only the increase of activity disappeared but no "spontaneous" activity could be detected, indicating that the glochidia were in the state of complete rest.

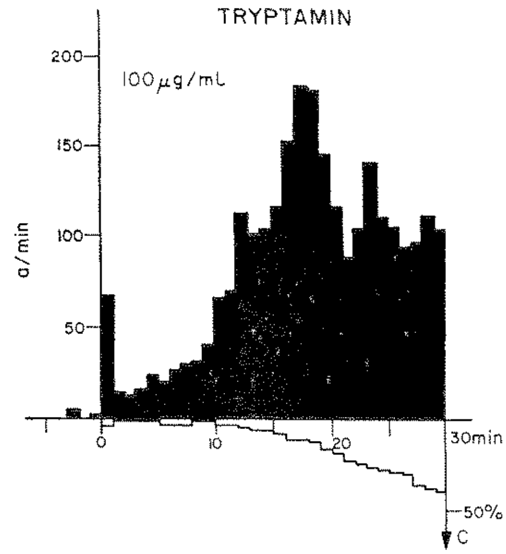

FIG. 4. Effect of tryptamin at 100 μg/ml on glochidial activity during spring. The lower curve shows the percentage of glochidia that are closed.

Effect of LSD, chlorpromazin and iproniazid

In adult molluscs the processes of excitation may be influenced by different chemicals, among them by LSD (Welsh, 1957; Hoyle & Lowy, 1956; Twarog, 1959; Koshtoyants & Rózsa, 1961a) and by iproniazid (Salánki, 1963). The effect of these agents are supposed to be connected with a probable mediator role

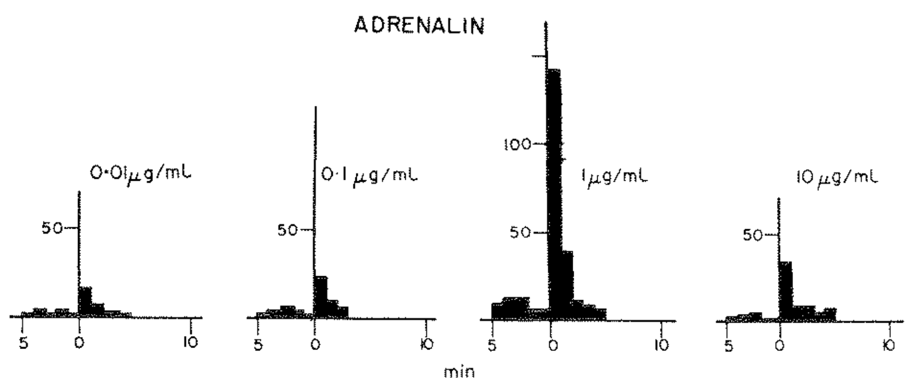

FIG. 5. The effect of adrenalin on the rhythmic activity of the glochidia. Abscissa, time in minutes; Ordinate, number of contractions per minute.

of serotonin. On the basis of our investigations we suppose that serotonin influences in glochidia the rhythmic activity in a different manner to tryptamin, and apart from the significant initial effect serotonin causes considerably less changes in activity. As a consequence the problem arises whether serotonin plays a similar

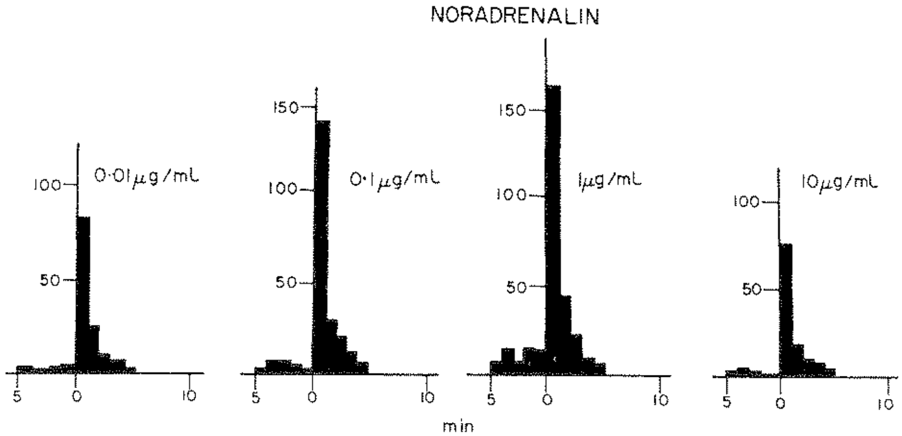

FIG. 6. Effect of noradrenalin on the rhythmic activity of the glochidia. Abscissa, time in minutes; Ordinate, number of contractions per minute.

role in the early stage of ontogenesis in *Anodonta* as in adult mussels. We investigated if LSD and chlorpromazin—known as serotonin-antagonists—and also iproniazid, the inhibitor of monoaminoxidase, cause any changes in the "spontaneous" rhythmic activity in glochidia and if they are able to influence the lasting increase of activity induced by tryptamin.

LSD in 90 μg/ml concentration caused a definite lasting increase of activity. It also induces the closure of the glochidia in more and more numbers and by the end of the tenth minute a third of them are already closed. When using LSD together with tryptamin the rate of the increase in activity is smaller than

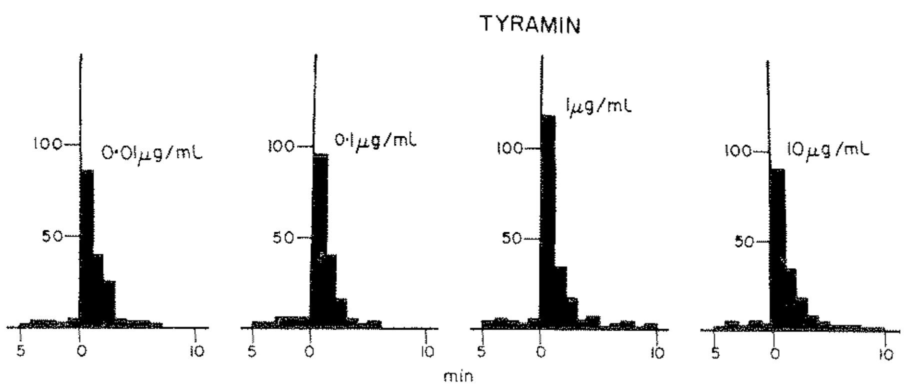

FIG. 7. Effect of tyramin on the rhythmic activity of the glochidia. Abscissa, time in minutes; Ordinate, number of contractions per minute.

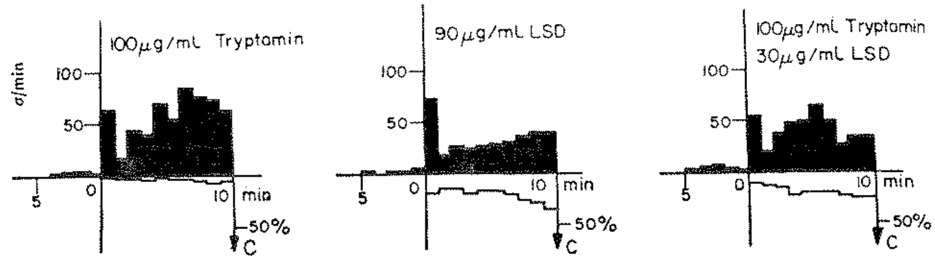

FIG. 8. Influence of LSD on the rhythmic activity of glochidia and on the activity-increasing effect of tryptamin. The lower curve shows the percentage of glochidia that are closed.

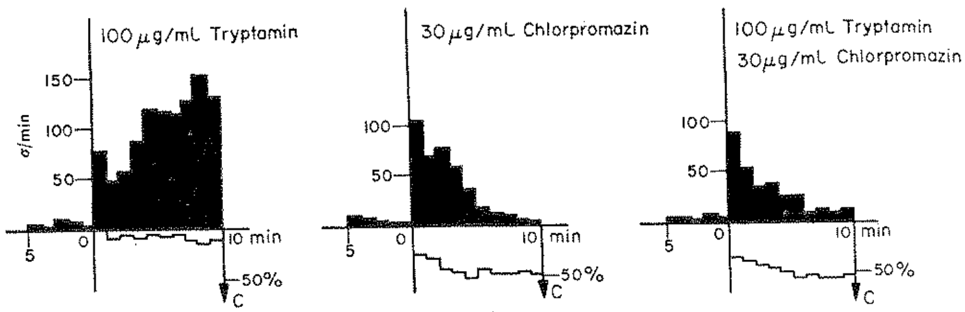

FIG. 9. Influence of chlorpromazin on the rhythmic activity of the glochidia and on the activity-increasing effect of tryptamin. The lower curve shows the percentage of glochidia that are closed.

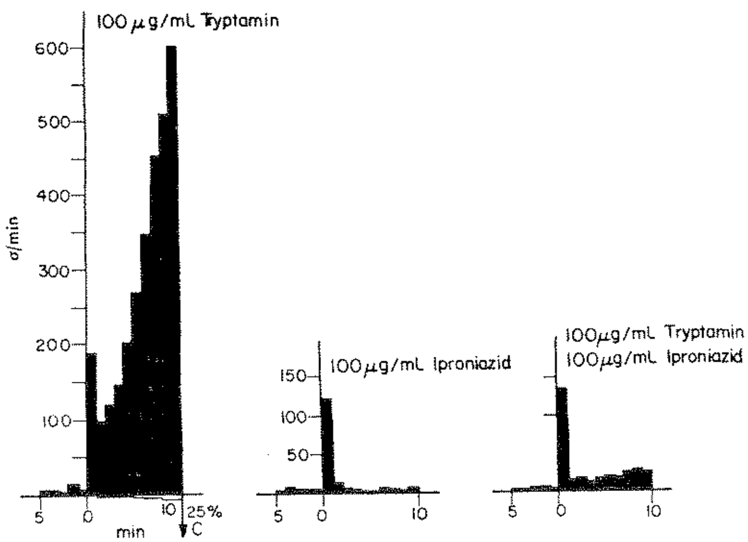

FIG. 10. The influence of iproniazid on the rhythmic activity of glochidia and on the activity-increasing effect of tryptamin.

in the case of the tryptamin control. But, especially in the first 6–8 min, it is higher than the increase of activity caused by LSD. The ratio of the closed glochidia in the case of 100 μg/ml concentration of tryptamin was 5% at the end of the tenth minute, whereas with LSD it reached 34%. Using them together the number of closed glochidia during the 10 min did not change compared to using only LSD (Fig. 8). The rate of the closure—giving the number of the closed glochidia at the end of each minute—is also demonstrated for each case. LSD inhibits the activity-increasing effect of tryptamin.

Investigating the effect of chlorpromazin on the rhythmic activity at 10–100 μg/ml concentrations, an initial increase of activity was detected, decreasing within 8–10 min to the level of the control. At the same time 50% of the glochidia closed. Using 30 μg/ml chlorpromazin and 100 μg/ml tryptamin together the increase of activity reached the level of the tryptamin control and it is significantly smaller than the sum of the increase of activity caused separately by tryptamin and by chlorpromazin. In this case immediately after the first minutes it was observed that the activity falls below the level of the tryptamin control and masks the effect of chlorpromazin. The curve shows that the number of the closed glochidia is greater than if using only chlorpromazin and comes close up to the sum of the effect caused separately by chlorpromazin and by tryptamin (Fig. 9). Thus chlorpromazin inhibits the effect of tryptamin and induces the closure of glochidia. The latter effect of chlorpromazin is not changed by tryptamin.

Controls with iproniazid (in concentrations 0·01 μg/ml – 100 μg/ml)—neglecting the first 2 min—did not cause any significant changes in activity. If used together with tryptamin, iproniazid inhibited the activity-increasing effect of tryptamin. Fig. 10 shows that in presence of iproniazid (100 μg/ml) the effect of tryptamin was fifteen- to twenty-fold less. The closure of the glochidia could not be detected either in the case of iproniazid alone or together with tryptamin. This shows that iproniazid inhibits the activity-increasing effect of tryptamin and also tonic contraction of the embryonic muscles in glochidia.

DISCUSSION

Investigating the motor activity in embryos of marine Gastropods it was found (Koshtoyants *et al.*, 1961) that they are extremely sensitive to serotonin. The activity-increasing effect of serotonin was also found in adult mollusc heart (Welsh, 1957; Erspamer & Ghiretti, 1951) and for the nervous elements (Koshtoyants & Rózsa, 1961a, b; Salánki, 1963). In our experiments on glochidia serotonin did not cause a long-lasting increase in activity and its smallest effective concentration was 0·1 μg/ml. The other chemicals were not able to increase the rhythmic activity of the glochidia below this concentration. L-tryptophan, 5 OH-tryptophan, adrenalin, noradrenalin and tyramin had an initial, activity-increasing effect which became definitely smaller after a few minutes and ceased completely within 10 min. Serotonin differs from the other agents partly by its striking initial effect, and partly by the high total value of the activity caused by it within the 10 min period.

The activity-increasing effect of tryptamin differs characteristically from the other agents investigated in our experiments. Although in the first minute the level of the activity in general is lower than in the case of other indole-alkyl-derivates and catecholamines, the effect does not take place within the 10 min but remains continuously even over 30 min. This long-lasting effect suggests that tryptamin is closer to the agents regulating the rhythmic activity of the glochidia under physiological conditions than serotonin. Catecholamines seemed to have no role in the regulation of the rhythmic activity, and the molecular protein receptors responsible for it show affinity to the indole-alkyl-amine derivates.

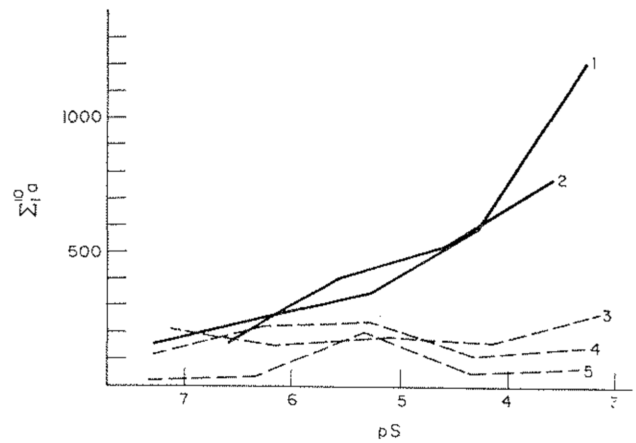

FIG. 11. Comparison of the effect of 1, tryptamin; 2, serotonin; 3, tyramin; 4, noradrenalin; 5, adrenalin. Abscissa, log of molar concentrations; Ordinate, total number of rhythmic contractions/10 min.

Comparing the effect of indole-alkyl-amines with the other investigated agents we found that the rhythmic activity can be raised by them to a higher level. This is demonstrated in Fig. 11. The abscissa gives the concentrations in logarithmic scale (pS), the ordinate shows the total number of contractions within the 10-min period. The effect of tryptamin and serotonin surpasses that of all other employed drugs between pS = 5–6.

The glochidial muscle has no innervation (Herbers, 1914) and the rhythmic activity can be interpreted as of myogenic origin. Therefore tryptamin seems to affect the embryonic muscle, producing a lasting increase of activity. It is very interesting to compare these results with Hill's data (1958) for the radula protractor in adult *Busycon canaliculatum*. Serotonin, in contrast to tryptamin causing a lasting rhythmic activity, did not influence it.

In the early state of development in *Anodonta* it is not serotonin but tryptamin that regulates the rhythmic activity of the embryonic adductor muscle. Under physiological conditions tryptamin is formed in the course of metabolism and maintains the contractions of the closer muscle. Tryptamin used under experimental conditions causes a long-lasting increase of activity.

The influence of LSD, chlorpromazin and iproniazid on the tryptamin effect is very noticeable. LSD and chlorpromazin are often mentioned as antagonists of serotonin, while iproniazid inhibits monoaminoxidase. Our investigations showed that separately employed, especially LSD, but also chlorpromazin, they cause a significant increase in activity, whereas used together with tryptamin they appear as tryptamin antagonists. So the conclusion might be drawn that LSD and chlorpromazin are not specific serotonin antagonists but in general the inhibitors of tryptamin receptors. It is possible that a competitive antagonism takes place giving a satisfactory explanation why these agents induce to a certain degree an increase of activity by themselves. Iproniazid alone does not increase the rhythmic activity of glochidia, but it antagonizes the tryptamin effect.

Comparing the influence of LSD, chlorpromazin and iproniazid on the tryptamin effect, we found inhibition in each case. In the presence of LSD and chlorpromazin one third to one quarter of the glochidia closed, but by adding iproniazid all glochidia kept open. This also verifies that LSD and chlorpromazin on the one hand and iproniazid on the other antagonize the effect of tryptamin in different ways. At present it would be difficult to give a satisfactory explanation for the cause of these differences.

No sign of equality can be drawn either between the effect of LSD and chlorpromazin. LSD alone causes an increase of activity over 10 min, and inhibits the tryptamin effect by decreasing the level of activity, whereas the lasting activity, corresponding to its control, remains unaffected. It might be connected with the fact that lysergic-acid-diethylamide itself is an indole derivate. Chlorpromazin alone causes an increase of activity stopping within 10 min, and together with tryptamin, the activity decreases to the level of the control during the same time. This fact obviously raises the question whether tryptamin could be identical with the physiological substance regulating the "spontaneous" rhythmic activity, as chlorpromazin inhibits only the tryptamin effect but has no influence on the "spontaneous" activity. At the present there is no definite answer, because it could be supposed that the role of the hormone could be different when released by the organism itself or when induced by outside.

Remarkable results were obtained in the early spring months with tryptamin. The fact that the tryptamin effect appears only after 4–6 min suggests that it may be not tryptamin itself responsible for the increase of activity, but one of its metabolic products. The different effect of tryptamin in the seasonal cycle could be connected with the fact that in spring the glochidia are in a more advanced stage of their development and there might be a change in the innervation or nature of the chemical control of the muscle.

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