The role of neurohumours in early embryogenesis

IV. Fluorometric and histochemical study of serotonin in cleaving eggs and larvae of sea urchins

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SUMMARY

The 5-hydroxytryptamine concentration changes insignificantly in the course of each of the first cleavage divisions, except at prometaphase when a sharp short-term fall occurs. According to the data obtained by the simplified gaseous formaldehyde method of Falck and Hillarp, 5-HT is present in all embryonic and larval cells up to the stage of prism. In plutei 5-HT is detected by this method only in the digestive tube, ventral ciliary band and possibly in pigment cells. According to the data obtained by the aqueous formaldehyde method, at the gastrula stage 5-HT is localized in the archenteron and vegetative ectoderm and at the stages of prism and pluteus in cells of the digestive tube, ventral ciliary band and, possibly, in pigment cells. The aqueous method gives negative results at pregastrular developmental stages.

On the basis of the data obtained a suggestion is put forward about the presence of two 5-HT pools in developing sea-urchin embryos and larvae. It is suggested that the first of them, not detected by the aqueous method and present in embryos beginning from the earliest developmental stages, performs intracellular regulatory functions whose character is discussed. The second pool detected by the aqueous method arises with the onset of gastrulation and appears to be related to morphogenetic processes.

INTRODUCTION

In previous papers (Buznikov, Chudakova & Zvezdina, 1964; Buznikov, Chudakova, Berdyheva & Vyazmina, 1968) it has been reported that regular and marked changes in the concentration of serotonin (5-hydroxytryptamine, 5-HT), catecholamines and acetylcholine (ACh) occur during the first cleavage divisions. A detailed analysis of these changes will help to establish how the neurohumours in question are involved in the processes of early embryogenesis. Such an analysis has already been carried out for ACh, noradrenalin and adrenalin (A) with fertilized eggs of Strongylocentrotus drobachiensis (Buznikov et al. 1968). It appeared necessary to obtained similar data for 5-HT as well. We expected that the comparison of dynamics of different neurohumours would lead...
to certain conclusions as to causes for the need of these substances during the first cleavage divisions.

The tasks of the present paper were not, however, limited by this aim. It is known that 5-HT, like, apparently, the other neurohumours, is functioning at all stages of sea-urchin development and not only during cleavage divisions (Buznikov, 1967; Buznikov et al. 1970; Gustafson & Toneby, 1970). New data concerned with this function could be obtained by means of histochemical determination of 5-HT in developing embryos. Such a study represented the second task of the present paper.

**MATERIALS AND METHODS**

The experiments were carried out mainly with cleaving eggs, embryos and larvae of a sea urchin *Strongylocentrotus intermedius* (Sea of Japan) and also *S. dröbachensis* (Barents Sea). The fertilized eggs were incubated under the optimal thermal conditions (20.5–21.5 °C and 7–8 °C, respectively). For fluorometric studies the material was homogenized with 0.5 N perchloric acid containing 0.25 % EDTA. In the course of cleavage divisions samples were being taken each 5 min. Some eggs were simultaneously fixed for subsequent determination of mitotic phases in total preparations (Melander & Wingstrand, 1953). In each series of samples eggs of the same female were used. In addition, a few samples were taken at later developmental stages of *S. intermedius* and *S. dröbachensis*. Prior to analyses, the precipitate was removed by centrifugation and the centrifugate was neutralized by 4 N Na₂CO₃ and purified in columns of the ion-exchange resin Dowex 50 (Manukhin & Poustovoitova, 1969). 5-HT was determined by fluorometry of the product of condensation with ninhydrin (Snyder, Axelrod & Zweig, 1965).

A histochemical study of 5-HT was carried out using fluorescence methods: the aqueous formaldehyde method and the simplified gaseous formaldehyde method of Falck and Hillarp (Sakharova & Sakharov, 1968, 1971). In the former case, embryos were incubated in 4 % formalin solution in sea water at 0 °C for 20 to 60 min., dried on slides in cold air, covered with paraffin oil and heated at 80 °C for 10–15 min. In the latter case, air-dried embryos were mounted in a mixture of paraformaldehyde powder and paraffin oil and heated at 80 °C under the coverslip. During this procedure paraformaldehyde was depolymerized and involved in the histochemical reaction. After the reaction was over (it was observed under the fluorescence microscope), the preparation was mounted in a pure paraffin oil. Control embryos were treated in the same way, omitting formalin and paraformaldehyde. Preparations were examined and photographed under the fluorescence microscope ML 2; conditions of filming and subsequent treatment of negatives were the same for all preparations. This allowed quantitative estimation of the value of fluorescence by the density of structures on negatives. This density was measured by the microphotometer MF 4; the resulting
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5-HT

\[< 30 \]

\[< 20 \]

\[< 10 \]

\[-5 \]

\[-1 \]

\[< \]

\[\text{Mitotic phases} \]

Fig. 1. Changes of 5-HT concentration in a sea urchin *S. intermedius* during the first cleavage divisions. 1, 2: results of fluorometric determinations in two series of fertilized eggs. 3: results of histochemical determination by the gaseous method. I = interphase; P = prophase; PM = prometaphase; M = metaphase; A = anaphase; T = telophase.

Measurements were given in arbitrary logarithmic units corresponding to the values of a galvanometer scale. The extent of histochemical reaction was estimated by the difference between the average results of experimental and control measurements.

**RESULTS**

(a) 5-HT in cleaving eggs

The results of fluorometric 5-HT determinations in cleaving eggs of *S. intermedius* are presented in Fig. 1. It can be seen that 5-HT is detected in all samples throughout the period from prometaphase of the first cleavage division till the end of the third division. The level of 5-HT suffers changes during the first cleavage divisions, these changes being similar in both series of fertilized eggs under study. The concentration of 5-HT is close to the value characteristic of unfertilized mature eggs (3–4 µg/10⁶ eggs) during almost the whole cell cycle and reliably decreases only at prometaphase. Such a decrease was observed during the first and second cleavage divisions but not during the third division. It should be mentioned in this respect that prometaphase predominated in none of the samples taken during the latter division.

When determining 5-HT histochemically in cleaving eggs, the simplified gaseous formaldehyde method in most cases gave positive results: yellow-green fluorescence characteristic of 5-HT was much more intensive than auto-
fluorescence in the control (Fig. 2c, d). As far as can be seen in total preparations, this fluorescence is inherent to all the cytoplasm; the fluorescence of the cell nucleus is somewhat more intensive.

The value of the histochemical reaction for 5-HT changes during the course of the cell cycle (Figs. 1, 2). These changes correlate distinctly with the changes in 5-HT concentration detected in fluorometric studies. A low 5-HT concentration in the samples taken at prometaphase of the second cleavage division coincides with a very weak statistically unreliable histochemical reaction on the corresponding preparations (Figs. 1, 2a, b). A high 5-HT concentration in the samples taken at metaphase–interphase of the second cleavage division corresponds to an intensive and statistically reliable histochemical reaction (Figs. 1, 2c, d).

The correlation is as if violated during the third cleavage division (Fig. 1). The violation appears, however, to be reconcilable: when determining 5-HT histochemically, a decrease of its concentration was detected at prometaphase of the third division. Recall that this period was not examined in our fluorometric study.

Histochemical determination of 5-HT by the aqueous formaldehyde method invariably gave negative results: fluorescence of the formalin-treated eggs was, as a rule, even weaker than autofluorescence in the control.

(b) 5-HT in embryos and larvae

It has been shown previously using an original biological method that following the period of synchronous cleavage divisions the level of 5-HT in embryos sharply decreases for a long time. A small increase is observed only at the stage of swimming blastula (approximately up to 50% of the value characteristic of synchronous cleavage divisions) (Buznikov et al. 1964). At later stages the level of 5-HT suffers rather large fluctuations and begins to increase steadily only from the prism stage.

The number of fluorometric 5-HT determinations carried out with embryos and larvae of *S. intermedius* and *S. dröbachiensis* is too small to construct such a detailed age curve. Therefore the main attention will be drawn to the results of a histochemical study, and first, to those obtained at the stages from early till late (mesenchymal) blastula.

When using the simplified gaseous formaldehyde method, the experimental embryos reveal, characteristic of 5-HT, a yellow-green fluorescence, more intensive than the autofluorescence in the control (Fig. 3a, b). This specific fluorescence is similar in all embryonic cells both by visual estimation and by the results of microphotometry of negatives. The intensity of histochemical reaction is very low at the early blastula stage (2 to 8 arb. log. units) and somewhat increases at the mid-blastula stage (20 arb. log. units) being, however, lower than that during synchronous cleavage divisions (Fig. 1). All this corresponds fairly well to the above-mentioned quantitative data obtained by the biological method.
Fig. 2. Formaldehyde-induced fluorescence in the fertilized eggs of a sea urchin, *S. intermedius*, during the second cleavage division. Gaseous method. Prometaphase: (a) formaldehyde-treated, (b) control. Metaphase: (c) formaldehyde-treated, (d) control.
Fig. 3. Formaldehyde-induced fluorescence at the stages of 2–4 blastomeres, early and mid-blastula (*S. intermedius*). A, Gaseous method: (a) formaldehyde treatment, (b) control. B, Aqueous method: (c) formaldehyde treatment, (d) control.
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Fig. 4. Formaldehyde-induced fluorescence in larvae of a sea urchin, *S. intermedius* (onset of gastrulation). Aqueous method: (a) = formaldehyde treatment, (b) = control. 1 = blastopore, 2 = primary mesenchyme.

From the early blastula stage till the onset of gastrulation the aqueous formaldehyde method gives, as earlier, negative results: fluorescence in experimental embryos is weaker than in the control (Fig. 3c, d). The situation changes with the onset of gastrulation when the aqueous method reveals the fluorescence, apparently due to 5-HT and localized in vegetative cells. Fluorescence can easily be seen (Fig. 4) in all cells of the forming archenteron. It is not excluded that a weak reaction for 5-HT is observed in primary mesenchyme cells as well, but this cannot be proved. All the other larval cells reveal no reaction for 5-HT when using the aqueous method. As far as can be judged by the results obtained with the simplified gaseous formaldehyde method, 5-HT is present in all cells of the early gastrula (Fig. 5a, b).

The aqueous formaldehyde method makes it possible to detect 5-HT in more-developed larvae of *S. intermedius* and *S. drobachiensis* as well. At the stages of mid and late gastrula and prism the specific 5-HT fluorescence is observed in all archenteron cells as well as in ventral ectoderm adjacent to blastopore (Fig. 5a, b).¹ The simplified gaseous formaldehyde methods reveal 5-HT in all cells at these stages as well as in early gastrula (Fig. 5c, d).

And, finally, in early and mid plutei (later stages were not examined) no differences were found between the results obtained by the two methods (Fig. 6). In both cases specific fluorescence is observed in all divisions of the digestive tube.

¹ These data coincide fairly well with the already published results of our preliminary experiments (Buznikov, 1971; Sakharova & Sakharov, 1971).
Fig. 5. Formaldehyde-induced fluorescence in sea-urchin larvae. A, *S. drobachiensis*, late gastrula. Aqueous method: (a) formaldehyde treatment, (b) control. B, *S. intermedius*, early and late gastrula and prism. Gaseous method: (c) formaldehyde treatment, (d) control. 1 = archenteron.
Fig. 6. Formaldehyde-induced fluorescence in larvae of a sea urchin, *S. intermedius* (stage of mid pluteus). A, Gaseous method: (a) formaldehyde treatment, (b) control. B, Aqueous method: (c) formaldehyde treatment. 1 = ventral ciliary band, 2 = digestive tube, 3 = pigment cells.
In the vegetative larval pole 5-HT is present only in cells of the ventral ciliary band. In all other larval structures (with the possible exception of pigment cells) 5-HT is detected neither by the aqueous, nor by the gaseous methods.

In summary, it can be mentioned that according to the results of a few fluorometric determinations the total 5-HT level suffers no marked changes at the postgastrular stages under study and is still lower than that at metaphase–early prophase of synchronous cleavage divisions.

**DISCUSSION**

5-HT is, thus, present in all cells of the embryo till the onset of gastrulation. From this moment on appear the first signs of heterogenous distribution of this substance and in plutei 5-HT is detected only in the digestive tube, cells of the ventral ciliary band in which the first neurons arise (Gustafson, 1970) and, maybe, in pigment cells.

The presence of 5-HT during the first cleavage divisions was established by two independent methods based, respectively, on reactions of 5-HT with ninhydrin and formaldehyde. A satisfactory coincidence of changes in 5-HT level determined by these methods (Fig. 1) witnesses to the reliability of the results obtained. Consequently, the negative results obtained when using the aqueous formaldehyde method during cleavage divisions and, in general, at pregastrular stages (Figs. 2, 3) cannot be accounted for by the absence of 5-HT in embryonic cells. These negative results cannot be accounted for by an insufficient sensitivity of the aqueous method as well. It has been suggested that the aqueous method has the same chemical background as the gaseous one and, therefore, they have, in general, comparable sensitivities (Sakharova & Sakharov, 1971).

It is possible that there are two types of 5-HT storage in sea-urchin embryos and larvae. One of 5-HT pools is not revealed by the aqueous method but is easily detected as a result of treatment of dehydrated material with depolymerized paraformaldehyde. This 5-HT pool is present in all cells of embryos and larvae up to the stage of prism. The second 5-HT pool is detected both by the aqueous and gaseous formaldehyde methods. It appears at the early gastrula stage and only in certain cell groups (archenteron, ventral ectoderm) and their derivatives.

The change of types of 5-HT storage is, possibly, related to the alteration of the functional role of this substance in the course of development. It is known (Buznikov, 1963, 1967, 1971; Buznikov et al. 1970) that during cleavage divisions 5-HT functions as a regulator of certain intracellular processes common to all cells. In accordance with this, 5-HT is present in all embryonic cells (first type of storage) and their sensitivity to antiserotonin drugs is approximately the same. The second type of 5-HT storage arises with the onset of gastrulation and is related to certain cell groups at the vegetative larval pole. According to extremely interesting new data (Gustafson, 1970; Gustafson & Toneby, 1970) it is these cell groups which are characterized by selective sensitivity to antiserotonin drugs.
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ACh

10

PM

Mitotic phases

Mitotic phases

Fig. 7. Changes in levels of acetylcholine (ACh), adrenaline (A) and 5-HT during the second cleavage division (scheme). Concentrations (µg/1 × 10⁶ eggs): (1) ACh: 5–7; A: 1.0–1.5; 5-HT: 3–4. (2) ACh: 2–4; A: 0.5–0.7. (3) ACh: < 2; A: ≤ 0.4; 5-HT: ≤ 1. Mitotic phases are designated as in Fig. 1.

and inhibitors of 5-HT synthesis. The character of effects described by these authors (inhibition of early gastrulation and primary mesenchyme formation) suggests that 5-HT of the second pool functions as a regulator of morphogenetic processes.

As to functions of 5-HT of the first pool, some new data can be obtained when analysing changes in 5-HT level during cleavage divisions. These changes markedly differ from those in the levels of ACh and catecholamines occurring at the same developmental stages. ACh and catecholamines are characterized by short-term rises of concentration on the background of low or zero level whereas for 5-HT a short-term fall on the background of a high and relatively constant level is characteristic (Fig. 7).

The dynamics of the first type allowed us to suggest trigger functions of ACh and catecholamines during cleavage divisions (Buznikov, 1967); the dynamics of the second type appears to exclude such a possibility for 5-HT. On the contrary, it can be suggested that 5-HT functions as an endogenous inhibitor of certain intracellular processes. These latter cannot as yet be precisely identified. However it is already clear that they: (a) should occur in cleaving sea-urchin eggs, most likely, immediately after the fall in 5-HT concentration, i.e. beginning with metaphase, and (b) should be directly concerned with cytokinesis since the processes of cytokinesis are characterized by the highest sensitivity to 5-HT antagonists (Berdysheva & Markova, 1967; Buznikov et al. 1970).

These initial requirements are satisfied by the processes of intracellular transport of chemical factors involved in determination and initial stages of cleavage furrow formation. The data concerned with the nature of these factors and character of their action are contradictory. However, it is known (Hiramoto,
1965, 1968; Kinoshita & Yazaki, 1967) that the intracellular transport of these factors in the fertilized sea-urchin eggs proceeds at metaphase. Therefore, it can be suggested that 5-HT of cleaving eggs inhibits the processes of intracellular transport of these factors; the fall of 5-HT concentration at prometaphase temporarily releases or decreases this inhibition. This working hypothesis is favoured by the fact that moderate concentrations of exogenous 5-HT delay the formation of cleavage furrows within each of the first cell cycles in the fertilized sea-urchin eggs (Buznikov et al. 1970).

On the basis of the above suggestion about the role of 5-HT in the processes of cytokinesis it can be expected that an experimentally induced untimely fall of 5-HT concentration will lead to a premature formation of cleavage furrow. We hope to verify this possibility in further experiments with the help of specific inhibitors of 5-HT synthesis. Further studies may also be aimed at a detailed analysis of intracellular 5-HT distribution at pre- and postgastrular stages of development.

РЕЗЮМЕ

Роль нейрогуморов в раннем эмбриогенезе.

IV. Флуорометрическое и гистохимическое изучение серотонина в дробящихся яйцеклетках морских ежей.

Концентрация серотонина (5-HT) изменяется по ходу каждого из первых клеточных циклов незначительно, если не считать кратковременного спада на прометафазе. По данным, полученными с помощью упрощенного гистохимического метода Фалька и Хилларпа, 5-HT присутствует во всех клетках эмбрионов и личинок морских ежей до стадии призмы включительно. На стадии плuteusa 5-HT обнаруживается этим методом только в пищеварительном тракте, вентральном поясе ресничек и, может быть, в пигментных клетках. По данным, полученным с помощью водного формальдегидного метода, 5-HT локализован на стадии гаструлы в архентероне и вегетативной эктодерме, а на стадиях призмы и плuteusa – в клетках пищеварительного тракта, вентрального пояса ресничек и, возможно, в пигментных клетках. На предгастрULAционных стадиях развития водный метод дает отрицательные результаты.

На основании полученных данных делается вывод о существовании у развивающихся эмбрионов и личинок морских ежей двух пуль 5-HT. Предполагается, что первый из них, не выявляемый водным методом и присутствующий у эмбрионов, начиная с самых ранних стадий развития, несет внутриклеточные регуляторные функции, характер которых обсуждается. Второй пул 5-HT, выявляемый как водным методом, так и упрощенным методом Фалька и Хилларпа, появляется с началом гаструляции и, по-видимому, связан с регуляцией морфогенетических процессов.
REFERENCES


(Manuscript received 19 July 1971)