The role of neurohumors in early embryogenesis

II. Acetylcholine and catecholamine content in developing embryos of sea urchin

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In a previous paper (Buznikov, Chudakova & Zvezdina, 1964) it has been reported that serotonin (5-hydroxytryptamine, 5-HT) may be involved in early embryogenesis in various groups of animals. This conclusion was confirmed by Baker’s recent publication (Baker, 1965) concerning 5-HT synthesis in *Xenopus laevis* embryos.

Some other low molecular weight substances, neurohumors or related compounds, are known to be synthesized in fertilized eggs as well. Acetylcholine (ACh) synthesis in sea-urchin eggs and embryos was demonstrated by Numanoi (1953, 1955, 1959, 1961). It seems possible that ACh can be synthesized in fertilized insect eggs as well (Morley & Schachter, 1963; Schachter, 1964). The synthesis of another neurohumor, dopamine (DA), in early insect embryos seems to be indisputable (Furneaux & McFarlane, 1965). However, in most cases changes in the level of neurohumors with age have not been studied.

In the present paper data concerning the change of concentration with age of ACh and catecholamines (adrenaline (A) and noradrenaline (NA)) in early sea-urchin embryos will be presented. We thought it would be of interest to investigate the suggestion that serotonin is not the only neurohumor synthesized in embryos of the sea urchin *Strongylocentrotus drobachiensis*. In addition, we compare the changes in the levels of various neurohumors with age.

**MATERIALS AND METHODS**

The experiments were carried out on developing embryos of the sea urchin *S. drobachiensis*. Most of the experiments were carried out at the Murmansk Marine Biological Institute (on the Barents Sea). After artificial fertilization, sea urchin eggs were incubated at 7 °C. During the time when we were taking...
samples some eggs were fixed with Carnoy's fluid to obtain total preparations (according to Melander & Wingstrand, 1953) for subsequent determination of mitotic phases. The results were expressed in μg of active substance (ACh, A or NA) per 10⁶ embryos.

(a) Estimation of acetylcholine

A dense egg suspension was washed twice with 0.04 N-HCl and then homogenized. Prior to homogenization 1 ml of 0.04 N-HCl and 5–6 ml acetone were added to 1 ml of suspension. The homogenate was incubated for 1 hr at 20–22 °C. The precipitate was removed by centrifugation. The supernatant was evaporated on a water-bath, the dry residue extracted with Locke solution diluted 2.4 times with double-distilled water. In some cases, the Loewi–Hellauer technique was employed for ACh extraction, which allows exclusion of evaporation (Loewi & Hellauer, 1938). It was found that the loss of active substance was quite small and reproducible and need not be taken into account. The extracts obtained were tested on leech dorsal muscle (Minz, 1955). Prior to the tests the muscle was treated with Locke solution diluted 2.4 times containing eserine or prostigmine (1·10⁻⁸ g/ml). Before the experiments 'dose-response' curves for ACh were plotted. The amplitude of muscle contraction produced by ACh served as a measure of the effect. In some cases to accelerate the relaxation, the muscle was washed with 5-HT solution (10⁻⁸ g/ml) as described by Poloni (1955) and Schain (1961). To test the specificity of the effects observed, nicotine was used, which is known to block cholinoreceptors of leech dorsal muscle (Minz, 1955). The threshold ACh concentration in our experiments was 5 × 10⁻¹⁰ to 1 × 10⁻⁹ g/ml. Some experiments were carried out on the Straub frog heart preparation. In this case only qualitative estimation of ACh could be made.

(b) Estimation of catecholamines

A dense suspension of the eggs was washed twice with 0.04 N-HCl, then homogenized in acidified ethanol (9 ml of 96 % ethanol and 1 ml of 0.25 N-HCl/ml of suspension). The homogenates were sealed in glass ampules and transported to Moscow. Prior to the analyses the homogenates were centrifuged and the supernatants evaporated on a water-bath or lyophilized. The dry residues were extracted with a 5 % solution of trichloroacetic acid, A and NA contents in extracts were determined by the highly specific colorimetric method developed by Manukhin (Manukhin, 1961; Manukhin & Vyazmina, 1967). The threshold values of A and NA which can be detected by this method were 0.001 and 0.01 μg respectively, accuracy ± 10 %. In some cases, simultaneously with analyses by Manukhin's method, the effect of the extracts on Straub frog heart preparations treated with atropine was tested. In some instances catecholamines were also identified by paper chromatography after preliminary purification of extracts on ion-exchange resin Dowex-50 (Manukhin, Pustovoytova & Vyazmina, unpublished data).
The time from taking samples to the beginning of analyses was 3–7 days: for 36 h the samples were kept at a room temperature and for the rest of the time in a refrigerator. Catecholamines are stable in acid solutions and any loss can be neglected.

RESULTS
(a) Acetylcholine

ACh was found to be present in mature unfertilized eggs of *S. drobachiensis* (Text-fig. 1). In the first minutes after fertilization the ACh concentration drops to zero. Then it rises quickly and reaches a maximum by 90 min after fertilization. This period coincides with the period of approach and contact of female and male pronuclei and with the rise of 5-HT concentration (Buznikov, Chudakova & Zvezdina, 1964). By the end of the second hour of development ACh practically disappears from the embryos.

Text-fig. 1. The changes of ACh content in developing embryos of the sea urchin *S. drobachiensis*. (1–4) First, second, third and fourth cleavage divisions respectively; (5) the motile blastula stage (hatching).

The first two cleavage divisions are accompanied by two almost equal (7–8 μg/10⁶ embryos) increases in ACh concentration. In the interval between them we did not find any detectable amounts of ACh in the samples. The formation of blastulae is characterized by two more rises of the ACh content, the second one coinciding with the onset of embryonal motility and with hatching. One analysis made during gastrulation revealed a rather low ACh level (0·8 μg/10⁶ embryos). At the stage of prism the ACh concentration increases again.
There are convincing reasons to propose that age changes of ACh level are much more complicated than would appear from the curve in Text-fig. 1, which is plotted from comparatively few experimental points. The thorough analysis of changes of ACh concentration with age which occur during the first mitotic cycles after fertilization confirms this suggestion (Text-fig. 2). Each point is the mean of 3–4 determinations. It has been found that in each of the first cell cycles there are not one but two peaks of ACh concentration. The first peak begins at the metaphase and reaches a maximum at anaphase. The second, more pronounced rise is less prolonged, and coincides with the late telophase, i.e. with the formation of the cleavage furrow. A two-peaked curve of this kind is typical at least of the first two mitotic cycles. The identification of ACh-like substance as ACh is supported by the following data:

1. Leech dorsal muscle, highly sensitive to ACh, does not contract under the influence of the majority of endogenous physiologically active substances which have been studied in this respect (Minz, 1955; Konzett & Riedler, 1963), but it does contract under the influence of the ACh-like substance.

2. Nicotine inhibits the sensitivity of leech dorsal muscle both to ACh and the ACh-like substance of sea-urchin eggs.

3. 5-HT abolishes the muscle contraction which is produced both by ACh and and the ACh-like substance.

4. Serum cholinesterase preparations (activity about 1 unit/ml) have been found to destroy specifically the active substance in extracts of sea urchin embryos. Extracts were treated with serum cholinesterase preparations for 40–60 min. at 12–14 °C. After such a treatment extracts lost their ACh-like effect.
The effect of extracts of mature unfertilized eggs of *S. drobachiensis* on the isolated frog heart.

Fig. A. The effect produced by the extract fraction, purified from ATP, on the heart previously treated with an adrenolytic. ↑, Time of introduction, ↓, time of removal, a, d, the extract fractions; b, atropine (cholinolytic); 5 × 10⁻⁶ g/ml; c, ACh, 1 × 10⁻⁷ g/ml.

Fig. B. The effect of an extract fraction, purified from ATP, on a heart which was not previously treated. A two-phase effect is observed, typical for solutions containing both ACh and A.
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both on leech dorsal muscle and on frog heart preparation. If the duration of incubation was less than 40-60 min, the extracts did not lose their potency. This suggests that cholinesterase acts on the active substance of extracts and not the pharmacological receptors of the test-objects. The effect of serum cholinesterase preparations on the ACh-like substance was abolished by eserine (1–5 × 10⁻⁵ g/ml).

5. The effect of the ACh-like substance, like that of ACh, on a frog heart has been found to be prevented by atropine (Plate 1).

6. Numanoi (1955) showed by means of paper chromatography that ACh and not other choline esters is present in sea urchin eggs.

The technique of extraction used in the present experiments allows us to determine total ACh. Thus we studied the true ACh synthesis in fertilized eggs but not the release of this substance from pre-existing inactive complexes.

We did not study the mechanism of ACh synthesis in fertilized sea-urchin eggs. As to the mechanisms of ACh loss during the first cell cycles, it does not seem to be connected with the presence of acetylcholinesterase (AChE). Using histochemical methods (Pearse, 1960) and a titrimetric micromethod, we found AChE activity in fertilized eggs only from the stage of motile blastula onwards. Similar results were reported previously by Augustinsson & Gustafson (1949).

(b) Catecholamines

Both NA and A are present in mature unfertilized eggs of S. dröbachiensis. The concentration of these substances varies from close to zero to a few tenths of a μg/10⁶ eggs. In the first 20-40 min after fertilization catecholamines are absent. Later they reappear, the variations in their concentrations being very sharp (from 0 to tens of μg/10⁶ eggs). Unfortunately, we have not been able to detect any regularity underlying these variations.

Much clearer results are obtained when examining the changes of NA and A contents during the second mitotic cycle. Four series of such analyses were carried out.

Despite significant individual variations, the changes during mitotic cycle were regular. They were more or less uniform in all the experiments, with two clearly pronounced rises of concentration at interphase and prophase–prometaphase (Text-fig. 3). There may also be a third rise at metaphase–anaphase, simultaneously with the first rise in ACh concentration (cf. Text-fig. 2). Between these rises the A concentration drops, sometimes to zero.

The NA content changes less regularly (Table 1). Increases in NA concentration are observed at interphase and at anaphase-telophase.

The results obtained in experiments with isolated frog heart preparations confirmed the presence of A in unfertilized sea-urchin eggs (Plate 1) and embryos. It has been shown that embryonic extracts affect the isolated atropinized frog heart in a similar way to A. This effect is completely prevented by adrenolytics. We also succeeded in identifying A and NA in extracts of fertilized sea urchin eggs by paper chromatography.
As judged by preliminary data, dihydroxyphenylalanine (DOPA) and dopamine (DA) are also present in fertilized sea urchin eggs. DOPA and DA may cause elevated values of A and NA when determined by Manukhin’s method. Even so it can be concluded that regular changes in catecholamine level (A and NA per se or together with DA and DOPA) do occur during the first cell cycles.

Text-fig. 3. Changes of A content in fertilized eggs of *S. drobachiensis* during the second mitotic cycle. T, telophase; I, interphase; P, prophase; Pm, prometaphase; M, metaphase; A, anaphase. The predominant mitotic phase is underlined. The arrows show the onset of the first and second cleavage furrow formation.

Table 1. Changes of NA content in fertilized eggs of *Strongylocentrotus drobachiensis* during the second mitotic cycle

<table>
<thead>
<tr>
<th>Egg sets</th>
<th>NA content (µg/10⁶ eggs)</th>
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<tbody>
<tr>
<td>I</td>
<td>— 0.54 0.79 — 0 — 0 — 1.64 —</td>
</tr>
<tr>
<td>II</td>
<td>0.45 2.54 0.32 2.30 0.53 — 0 1.02 0</td>
</tr>
<tr>
<td>III</td>
<td>— 0.55 1.25 0.11 0 — 0 — — —</td>
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Abbreviations as in Text-fig. 3.

The method of extraction used in the present experiments is such that total catecholamine content is estimated. Thus in this case, as that of ACh, we observe total synthesis of neurohumors in fertilized eggs rather than the release of neurohumors from inactive pre-existing complexes.
We have not studied the pathways of synthesis and inactivation of catecholamines in early embryos. However, inactivation of catecholamines is unlikely to be due to monoamine oxidase action. At present it is not known whether catecholamine inactivation is caused by other enzymes or by their elimination into the surrounding water.

One of the catecholamines, NA, has been found in fertilized eggs of the loach *Misgurnus fossilis*. Colorimetric and chromatographic methods have been used in these experiments; changes of NA level with age have not been studied.

**DISCUSSION**

Fertilized eggs and early sea-urchin embryos have been shown to synthesize a number of neurohumors: serotonin, acetylcholine, adrenaline, noradrenaline and, possibly, dopamine; loach embryos synthesize serotonin and noradrenaline. This is the main conclusion drawn from the results obtained in our previous and present experiments.

Regular changes of concentrations during the first cell cycles are likely to be characteristic of all neurohumors studied. It should be noted that the curves presented in the paper can reflect both the rate of the synthesis and that of the removal of neurohumors. This makes their analysis difficult (Text-figs. 2, 3). For example, we do not know whether the rises of the ACh level at the anaphase and telophase (Text-fig. 2) are caused by an increase in synthesis or a slowing of removal of this substance. The first possibility seems to be more probable. However, irrespective of the cause of the sharp rises in the level of the neurohumors, the fact that these rises do occur suggests that these substances play a part in the regulation of the first cell division cycles.

One cannot exclude the possibility that the real ACh content in sea-urchin embryos is higher than measured owing to the presence of significant amounts of ATP in the samples. As ATP level in sea urchin embryos remains constant throughout the period of cleavage divisions (Chambers & Mende, 1953; Hultin, 1957; Nilsson, 1961; Taguchi, 1962; Epel, 1963; Zotin, Milman & Faustov, 1965) this difference between real and experimental ACh content should be the same in all the samples. Therefore this difference could not have a fundamental influence on the character of the age curves.

Further confirmation that neurohumors do play a regulatory role in this case is given by the concentrations of these substances. Concentrations of neurohumors in sea-urchin embryos are much lower than those of usual metabolites and similar to the concentrations of neurohumors in adults, where they do play a regulatory role.

The relationship between increases of neurohumor concentrations and events during cell division is confirmed by the data obtained on infusoria *Tetrahymena pyriformis* (Sullivan & Sullivan, 1964). Experiments on synchronized cultures of *T. pyriformis* have shown that the ACh content rises rhythmically during cell
divisions. Our experiments on the action of phenothiazine derivatives on fertilized eggs of different sea-urchin species give confirmatory evidence for direct participation of neurohumors in the regulation of cell division (Buznikov, 1966, 1967; Buznikov & Berdycheva, 1966). It should be noted that the character of the intercellular regulatory functions of these substances is still not quite understood.

**SUMMARY**

1. Acetylcholine (ACh) is found in unfertilized eggs of sea urchin *S. dröbachiensis*. Immediately after fertilization the level of this substance drops sharply, but in the course of cleavage divisions and at the stages of early blastula, late blastula and prism the concentration shows transient sharp increases.

2. In the course of the second mitotic cycle, two increases of ACh content are observed in fertilized eggs of *S. dröbachiensis*. The first rise occurs at metaphase-anaphase and the second, more pronounced, during the formation of the cleavage furrow.

3. Adrenaline (A) and noradrenaline (NA) are also found in unfertilized eggs of *S. dröbachiensis*. After fertilization, sharp changes in the content of these substances occur which we have not followed in detail.

4. Increases in A concentration are observed at interphase and prophase-prometaphase. The content of NA increases at interphase. It is suggested that such changes in the level of ACh, A and NA take place during both of the first cleavage divisions and that these substances play a part in the regulation of cell division.

**Резюме**

1. Ацетилхолин (АХ) обнаружен в зрелых неоплодотворенных яйцеклетках *S. dröbachiensis*. После оплодотворения уровень этого вещества резко снижается. Подъемы концентрации АХ отмечены на 90-й минуте после оплодотворения, а также на стадиях дробления, ранней бластулы, подвижной бластулы и призмы.

2. Адреналин (А) и норадреналин (НА) также обнаружены в яйцеклетках *S. dröbachiensis*. После оплодотворения наблюдаются резкие изменения концентрации этих веществ, детально не изученные.

3. Во время второго митотического цикла в оплодотворенных яйцеклетках *S. dröbachiensis* наблюдается два подъема концентрации АХ. Первый из подъемов происходит во время метафазы анафазы, а второй, больший по абсолютной величине — во время образования борозды дробления. Подъемы концентрации А также наблюдаются дважды — во время интерфазы и профазы-прометафазы, а подъем концентрации НА — во время интерфазы. Предполагается, что такие изменения уровней АХ, А и НА происходят во время каждого из первых делений дробления.
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REFERENCES


(Manuscript received 21 April 1967, revised 1 February 1968)