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Acid-Soluble Nucleotides during Early Embryonic Development of the Sea-Urchin *Paracentrotus lividus*

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Trichloroacetic acid extracts from unfertilized eggs and from different stages during the early development of the sea-urchin *Paracentrotus lividus* were analysed for nucleotides and for inorganic, acid-labile and acid-stable phosphates.

Fertilization was followed by a drop in the content of inorganic phosphate and a rise in the acid-labile and acid-stable phosphates. The acid-labile phosphate afterwards showed cycle variations during the first five divisions with an increase in the concentration during the early part of mitosis and a fall during the later part. The inorganic- and acid-stable phosphate fractions varied inversely with the acid-labile fraction.

The total nucleotide content did not vary appreciably during the first hours of development but showed an appreciable decrease 20 hours after fertilization. The individual compounds, however, varied, the greatest changes taking place in connexion with the fertilization and around the 16–32 cell stages.

During the last few decades the phosphorus metabolism of the sea-urchin embryo has been the subject of several investigations. Zielinski [1] found no change to occur in the inorganic phosphate content during the first cleavages of *P. lividus;* neither did he find any change in the amount of organic pyrophosphates. Similar results were obtained by Örström and Lindberg [2], who were unable to demonstrate any changes in inorganic and acid-labile phosphates following the fertilization of the sea-urchin egg. However, in 1949 Chambers and White [3], who used eggs of *Asteria forbesii* and *Strongylocentrotus dröbachiensis* found that inorganic phosphate disappeared and that acid-labile phosphate increased upon fertilization. According to Chambers and Mende [4], the reaction following fertilization was the incorporation of inorganic phosphate into arginine phosphate. Using enzymatic methods these authors [5] were unable to demonstrate any variation of the amounts of different adenosine nucleotides in various stages cf the first cell cleavage cycle. Recently

Hultin [6], who used chromatographic methods, measured the amounts of ATP *, AMP, UTP and UMP during the development of *Psammechinus miliaris*. He analyzed stages from the fertilization to the plutei stage and found a gradual decrease in the triphosphate content, while the monophosphates increased slightly.

This paper is concerned with determination of the amounts of different phosphate fractions, especially the different nucleotides, during the first 8 h of the embryonic life of the sea-urchin *P. lividus*. The occurrence of these substances in the unfertilized egg was reported in a previous paper [7].

MATERIAL AND METHODS

The sea-urchin species used was *Paracentrotus lividus* collected at *Station Biologique, Roscoff*. The eggs were shed in beakers containing sea water, freed from debris by filtering through gauze and washed four times by decanting with sea water. Dilute suspensions of the eggs were fertilized and allowed to develop in large glass vessels at 16°C. The culture was gently agitated by stirring with a motor driven glass rod. The development was followed by microscopic observation and only cultures with 100 % fertilization and which cleaved synhronously to at least the 32-cell stage were used for analysis. Aliquots were withdrawn from the cultures at different stages in the development, extracted with trichloroacetic acid in the way described previously [7]. A small part of the extract was used for phosphorus-analysis, the main part was freeze-dried and stored at −25°C until further analysis.

Analytical procedures, ion-exchange and paperchromatographic methods were the same as used before [7].

RESULTS

The main purpose of the investigation was to ascertain whether the amounts of the different nucleotides in the unfertilized sea-urchin egg underwent any variations during its development. However, the extract was also first analysed for its content of different phosphate fractions.

Only minor differences were found in the total amount of acid-soluble phosphates. But when the phosphate content was differentiated into phosphate determined as inorganic phosphate (P_i), acid-labile phosphates (P_2 and P_{10}) and acid-stable phosphate, variations were found within each group (Fig. 1). In agreement with the finding of Chambers and Mende [4], the amount of P_i decreased after fertilization, but it increased again before the first cleavage. Cyclic variations with a fall in the P_i content immediately after the cleavage and then an increase before next division recurred, at least during the first five cell divisions.

The fall in the P_i content following fertilization was compensated by an increase in the acid-labile phosphate fractions. Both the P_2- and P_{10} fractions increased after fertilization and afterwards varied inversely with the P_i content. The acid-stable phosphate content also increased upon fertilization, but then varied approximatively with the P_i content up to the 32-cell stage.

* ATP = 5′-adenosine triphosphate; GTP = 5′-guanosine triphosphate
ADP = 5′-adenosine diphosphate; GDP = 5′-guanosine diphosphate
AMP = 5′-adenosine monophosphate; GMP = 5′-guanosine monophosphate
UTP = 5′-uridine triphosphate; CTP = 5′-cytidine triphosphate
UDP = 5′-uridine diphosphate; CDP = 5′-cytidine diphosphate
UMP = 5′-uridine monophosphate; CMP = 5′-cytidine monophosphate
DPN = diphosphopyridine nucleotide; RNA = ribonucleic acid

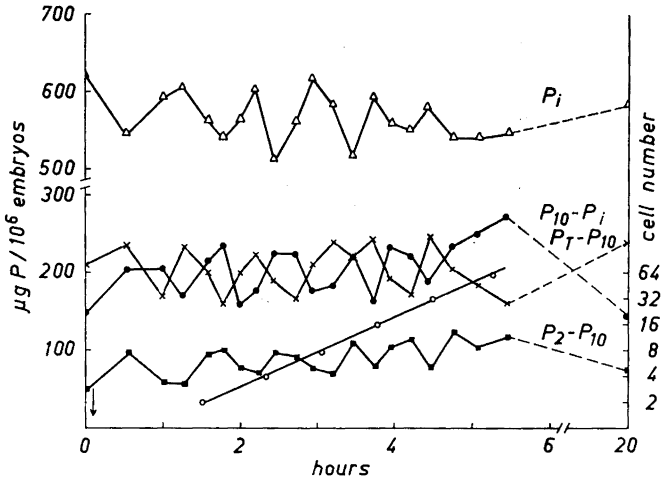

Fig. 1. Variation in the different phosphate fractions during the development of *P. lividus*. (△—△ P_i, ●—● $P_{10}—P_i$, ■—■ $P_2—P_{10}$, ×—× $P_T—P_{10}$, O—O cellnumber). The arrow indicates the time of fertilization.

After the 64-cell stage the synchronicity of the cultures began to disappear and the analysis was therefore not extended beyond this stage.

Like the total amount of the acid-soluble phosphates, the nucleotide content, as measured by the optical density at 260 mμ, did not vary to any greater extent during the period investigated. However, the ultraviolet absorption of the extract is not a true measure of the nucleotide content, as it has been shown [7] that about half of the optical density at 260 mμ in the extract of the unfertilized egg can be ascribed to non-nucleotide compounds. This also holds for the extract from the embryonic stages studied. However, when the amount of optical density at 260 mμ retained on Dowex-1, was taken as a measure of the nucleotide content there were only minor differences in the total nucleotide content of the embryo during the first 8—10 h (Table 1).

Fig. 2 shows a schematic picture of the main nucleotides in the unfertilized egg, in the embryo 8 h after fertilization when it contains about 300 cells, and in the early gastrula 20 h after fertilization. The unfertilized egg contained

Table 1. Amount of ultraviolet absorbing substances measured at 260 mμ in acid extracts from different embryonic stages of *P. lividus*, and the amount of the substances adsorbed on Dowex-1 from the same extracts.

	$E_{260}/10^6$ embryos									
Time in hours	0	1	2	3	4	5	6	7	8	10
Total extract	69	66	66	69	67	66	67	70	67	65
Adsorb.on Dowex-1	34	33	32	32	34	33	32	31	33	31

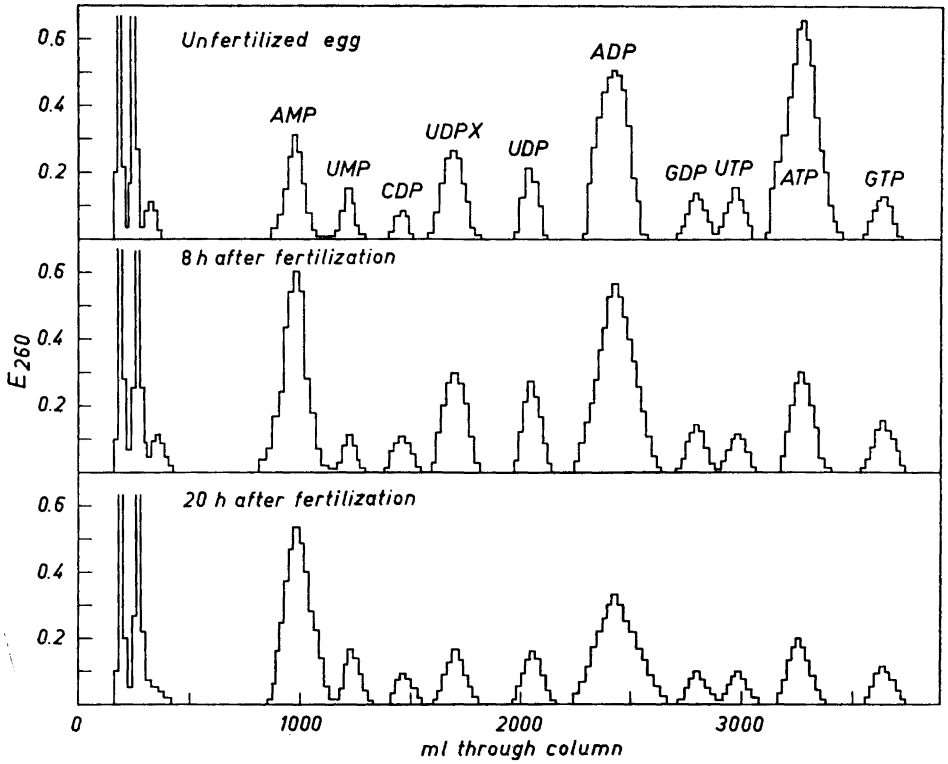

Fig. 2. A schematic picture of ion-exchange chromatograms of trichloroacetic acid extracts from unfertilized *P. lividus* eggs, from embryos 8 h, and 20 h after fertilization. Column: Dowex-1-formate (200—400 mesh, 1 × 20 cm). Elution solution: the formate system previously used [7].

appreciable amounts of ATP and ADP, but only a small amount of AMP. At 8 h after fertilization the ATP content was markedly reduced; the AMP content, increased; and the amount of ADP, largely unchanged. At the time of gastrulation 20 h after fertilization all fractions had diminished, but in relation to ATP and ADP the AMP content showed a further increase.

The other nucleotides demonstrated in the chromatograms were present in such small amounts that no variations in their concentrations could be seen in the figure. The three peaks in the beginning of the chromatograms contained several cytosine- and adenine derivatives some of which were not identified. They contained, amoung other substances, CMP and DPN.

A more detailed picture of the nucleotide content during the first hours of development of the sea-urchin embryo is shown in Fig. 3. The amounts of ATP, ADP, *etc.* were determined at different times up to the 64-cell stage 5.5 h after fertilization and then at 8 and 20 h after fertilization.

Regarding the adenine nucleotides (Fig. 3a), fertilization was followed by a considerable drop in the ATP content, which diminished from 6.5 to 4 μmoles

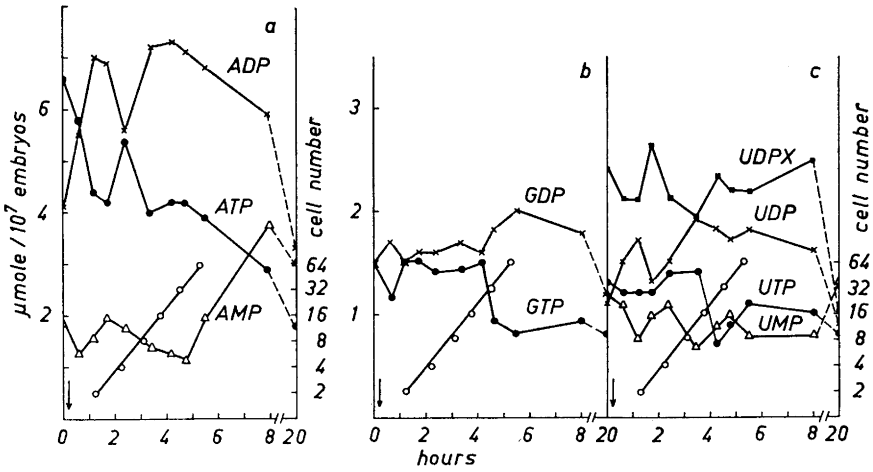

Fig. 3. Variation of adenosine-, guanosine- and uridine nucleotides during the development of *P. lividus*. The arrows indicate the time of fertilization. (O—O cellnumber.)

per 10^7 embryos, a concentration obtained at the time of the first cell division. Between the first and the second division the ATP content increased again to a value of 5.5 µmoles per 10^7 embryos. From the 4-cell stage to the 16-cell stage the amount of ATP then decreased to 4 µmoles. From this stage to the 64-cell stage the content remained fairly constant, but then slowly fell to 3 µmoles at 8 h after fertilization. In the gastrula the ATP content was further reduced to 2 µmoles per 10^7 embryos, *i.e.* a value less than a third of the initial concentration.

The ADP content varied inversely with the ATP content during the first cleavages. The concentration rose from 4 to 7 µmoles, then fell to 5.5 µmoles per 10^7 embryos and rose again to 7 µmoles per 10^7 embryos. After the 16-cell stage the ADP content showed a fall corresponding to that of the ATP content. The ADP content in the early gastrula was only slightly lower than in the unfertilized egg.

The AMP content of the unfertilized egg was low compared with ATP and ADP, and it did not vary appreciably during the first cleavages. However, after the 32-cell stage it showed a steady increase, and at 8 h after fertilization the AMP content was doubled. The amount was somewhat reduced in the early gastrula.

Of the guanine compounds, only GTP and GDP were estimated, the amount of GMP being too small to allow of a reliable analysis. Like ATP, the GTP content dropped in connexion with fertilization, but rose again to the initial value before the first cell division and then remained almost constant to the 32-cell stage. This stage was followed by a fall in the GTP content from 1.5 to 0.8 µmoles per 10^7 embryos. GDP varied roughly inversely with the GTP content (Fig. 3b).

Fig. 3c shows the variations in the uracil nucleotides. Besides the identified UTP, UDP and UMP fractions, a hitherto not completely identified frac-

tion, UDPX, was also estimated. It is an UDP derivative, which on hydrolysis yielded several amino acids including alanine, serine and glutamic acid.

Of the uracil compounds, UTP was affected least by fertilization, its concentration remaining nearly constant during the first cell-divisions. At the 16-cell stage the amount fell to 50 % of the initial value, but then slowly rose again. UMP showed rhythmic variations with the highest concentration in the unfertilized egg, in the 4-cell and 32-cell stages. The variations in the UDP and UDPX content showed a roughly inverse relationsship with a rise in the amount of UDP following fertilization. In connexion with the first cleavage the amount fell but increased again and reached a maxium immediately before the 16-cell stage. The UDP content then slowly decreased.

DISCUSSION

The fertilization of the sea-urchin egg starts a variety of chemical and metabolic reactions, which are the bases of embryonic development. The large number of different reactions make this process very complex and not easy to analyze. However, in the sea-urchin the problem is somewhat simplified by the fact that the embryo does not take up any food during the first period of its life. The changes found in the amounts of different substances can therefore be related entirely to reactions within the embryo. It should, however, be observed that the variations in the amount of a substance is not a measure of the rate of a single reaction but the resultant of both catabolic and anabolic reactions. The resultant may take the form of cyclic variations in the amount of a substance if the catabolic reaction is predominant at one time and the anabolic reaction at another time. The cycles may extend over several mitoses, as was shown by Kavanau [8] for free amino acids and by Bäckström [9,10] for some —SH containing substances and to some extent even for RNA. Fluctuations coinciding with the mitosis were shown by Saki and Dan [11] to be true for TCA-soluble —SH groups, but the best known examples of this type of variation is probably the rhythmic variations in oxygen-uptake found by Zeuthen[12,13] in marine eggs. In the present work both types of variations were found to occur during the early development of the embryo. The variations in the amounts of the different phosphate fractions coincided with phases of the mitotic cycle, while the nucleotide fractions showed cyclic variations covering several cell divisions.

The energy necessary for the development is generally assumed to be derived mainly from the oxidation of carbohydrates stored in the unfertilized egg [1,2]. Lindahl [14] has shown that the respiration, which in the unfertilized egg had a *RQ* of 0.73 indicating fat oxidation, increased after fertilization and further that the increment in respiration had a *RQ* of 1.0 suggesting a carbohydrate utilization. A decrease in the amount of carbohydrates following fertilization was found by Örström and Lindberg [2] and by Chambers and Mende [4].

The energy released on oxidation of carbohydrates is mediated to synthetic processes by means of phosphorylated metabolic intermediates. By determining the amount of the different acid-labile and acid-stable phosphates, it

should therefore be possible to obtain a picture of the energy expenditure during the development of the embryo.

However, as the embryo contains several phosphate esters, estimation of every one of them would be very time-consuming. Therefore, in this work a compromise was made; the acid-soluble phosphates were differentiated by phosphorus analysis into orthophosphate (including organic phosphates, which are hydrolysed to orthophosphate during the analytical procedure), acid-labile phosphates and acid-stable phosphates. One group of the phosphates, the acid-soluble nucleotides, was then analyzed in further detail.

Fertilization of the sea-urchin egg is accompanied by appreciable changes in the different phosphate fractions; a decrease in the orthophosphate content and an increase in the acid-labile phosphate fractions. This is in accordance with the work of Chambers and Mende [4], who showed that the labile phosphate formed was arginine phosphate. This substance is analysed as orthophosphate already after hydrolysis for 2 min. and is therefore easily distinguished from the acid-labile nucleotides, which need hydrolysis for 10 min. to split off inorganic phosphate.

The changes initiated by the fertilization were followed by rhythmic variations in the phosphate fractions for at least the first five mitoses (Fig. 2). The increase in acid-labile phosphate during the first stage of mitosis implies that during this period an energy depot is being built up to be used during the cleavage of the nucleus and the cytoplasm. The variations are synchronous with the variations in the oxygen uptake shown by Zeuthen [12,13] to be valid, at least during certain conditions, for the sea-urchin embryo. The oxygen uptake increased during the prophase and decreased during the later mitotic stages.

Among the nucleotides, the adenosine, guanosine and uridine compounds were studied for any variations in amount (Fig. 3). The adenosine derivatives occurred in the largest amount and they also showed the clearest picture during the development of the sea-urchin (Fig. 3a).

The adenosine nucleotides are generally thought to be connected with the conversion of energy in biological systems and variations in these substances may therefore indicate when substantial changes in energy are taking place. The first change in the nucleotide content was found to be related to the fertilization. This process was accompanied by a large and rapid fall in the ATP content and a corresponding rise in the ADP content. These changes may at least in part be attributed to the use of ATP for the synthesis of arginine phosphate.

$$\text{Arginine} + \text{ATP} \rightleftharpoons \text{Argininephosphate} + \text{ADP}$$

The fall in the concentration of inorganic phosphate depends then on the rephosphorylation of ADP by glycolysis and/or oxidative phosphorylation.

$$\text{ADP} + \text{P}_\text{i} \rightleftharpoons \text{ATP}$$

The fertilization and the first cleavage required a considerable amount of high energy compounds, as is seen from the large decrease in the ATP-content during this period.

This first period of expenditure of energy was followed by a short period — around the second and third divisions — with a net synthesis of ATP. However, the increase in the ATP-content was only temporary and during the rest of the period studied, the amount of ATP and of ADP showed a steady decrease indicating that the embryo is in a period requiring a large supply of energy with a maximal function of the ATP utilizing systems. The preponderance of the energy consuming reactions is not only seen in the decrease in the ATP- and ADP stores but also in the increase of the AMP content. When the ATP concentration is low enough, even the pyrophosphate bond in the ADP molecule can be utilized for energy transfers. For this purpose ADP is dismutated by adenylatekinase to ATP and AMP.

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$

Since ATP constantly being consumed the reaction is shifted to the right resulting in an increased AMP concentration.

The enhanced need for ATP is not only due to synthetic work for development, but also to the fact that after the hatching a large amount of energy is needed for ciliar movement in the swimming embryo.

Of the guanosine phosphates only GTP and GDP occurred in amounts large enough to allow of quantitative estimation. The largest changes in these compounds took place in connection with the fertilization and around the 16—32 cell stages, the early blastula. As mentioned above, the fertilization was followed by an increased respiration and a further increase occurred in the early blastula, as shown by Lindahl [15] and Borei [16]. According to Kavanau [8] the first intensive protein synthesis also begins at this stage. It is known that GTP is a cofactor in transphosphorylations in connexion with oxidation of some intermediates in Krebs' cycle [17,18] and that it is also required for some steps in protein synthesis [19]. One may therefore assume that the fall in the GTP concentration at the periods indicated are related to the increase in these activities.

The most complex picture was shown by the uridine derivatives. It is well known that these compounds are of greatest importance in the carbohydrate metabolism in which they take part as coenzymes and carrier of sugars and sugar fragments. Since the carbohydrate metabolism supplies the major part of the ultimate energy for the development [1,2,14] there should reasonably be wide variations in the different uridine compounds.

Immediately after fertilization, the UDP content increased, but as the UTP content did not change at that time, the increase was not caused by dephosphorylation as in the case of ATP—ADP. Instead, it seemed probable that UDP was released from some other type of nucleotide in connexion with the drop in reducible carbohydrates. The UDP content reached a maxium just before the first cell division was completed and then fell to the original value. A new maximum was, however, reached at the 16-cell stage. At this stage there also started a period of intensive metabolism, as judged by the steep increase in the oxygen consumption found by Lindahl [15]. The increase in the oxygen consumption lasted for about 3 h, and during approximatively the same time the UDP content persisted at a level nearly twice as high as the initial.

The compound from which UDP could be released or into which it could be incorporated may be the fraction called UDPX. This assumption is supported by the fact that the two compounds varied inversely indicating some sort of interaction. Unfortunately UDPX has not been fully identified. It might be a complex fraction containing several very similar nucleotide compounds, but which all contained UDP as the nucleotide moiety.

The UTP content did not show any appreciable change until the 16-cell stage was reached. At that stage, however, it rapidly dropped to half the amount originally present. The drop in the UTP content coincided with that of GTP and may be due to the increase in the metabolism at this stage. It may also be caused by incorporation of nucleotides into RNA, which, according to Bäckström [10], is rapidly synthesized at this time.

Cytidine nucleotides have not been discussed since only CMP occurred in amounts large enough for quantitative measurements. There were, however, also fractions which, judging from the positions in the ion-exchange chromatogram, should be CDP and CTP, respectively. Another cytidine derivative also obtained in too small an amount to allow of accurate measurement was CDP-choline. This compound probably plays a role in the lipid metabolism. Unfortunately the amount of embryonic material available was unsufficient to permit further analysis for this and other cytidine compounds.

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