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DNA and RNA Content of the Dog Salmon Egg During Early Embryonic Development

By

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(With 11 Text-figures and 2 Tables)

Studies on the nucleic acids during embryonic development revealed that there is a cytoplasmic reserve of deoxyribonucleic acid (DNA) in various invertebrate and vertebrate eggs (Elson and Chargaff, 1952; Brachet, 1954; Schwartz, 1970; *etc.*). This reserve is used for the embryonic nuclei during early development. It is thought that the starting time of DNA synthesis depends on the quantity of the cytoplasmic reserve. For instance, DNA synthesis begins in the early cleavage stage of sea urchins and the cytoplasmic reserve of DNA is very small (Elson *et al.*, 1954). In amphibians, active synthesis of DNA appears at the late blastula or early gastrula stage and the DNA reserve within the cytoplasm is very large (Chen, 1960). It could be supposed, therefore, that the amount of DNA reserve is proportional to the egg size and that DNA synthesis is initiated in the early stages of development in animals having smaller eggs than in those having larger ones (Solomon, 1957; Kong *et al.*, 1971). As to the behavior of ribonucleic acid (RNA) during embryonic development, there is a sharp difference between sea urchin and amphibian eggs. RNA content remains constant until the pluteus stage in sea urchins (Elson *et al.*, 1954) but increases significantly with the process of differentiation in amphibian embryos (Krugelis *et al.*, 1952; Chen, 1960). In view of the difference found in sea urchins and amphibians, it is desirable to know the quantitative change of nucleic acids during embryonic development in different organisms. Scant information is available on the absolute amount of DNA and RNA found in teleost embryos at successive stages of development. Perhaps this is partly due to the difficulty of extracting nucleic acids from lipid- and protein-rich eggs (Hagenmaier, 1969). This paper describes the method for extraction of nucleic acids and their quantitative change during early embryonic development of the dog salmon.

Material

Materials used in the present study were derived from six males and six females of the dog salmon, *Oncorhynchus keta*, which were captured at the Chitose Hatchery in Hokkaido.

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A long cut was made on the belly and ripe unfertilized eggs were taken in a vessel. After several washings with isotonic Ringer's solution (Yamamoto, 1967), the eggs were artificially inseminated in the same solution. Two minutes later, the eggs were thoroughly washed with a large amount of isotonic Ringer's solution to reduce the number of excess spermatozoa adhering to the egg surface. It was ascertained that the spermatozoa were nearly all removed from the egg surface by this procedure, except in the micropylar region where the excess spermatozoa filled the micropylar canal. These eggs were designated as unactivated eggs in this study. The fertilized eggs were immersed in water. As previously reported (Yamamoto, 1967), the salmon egg is activated and starts to develop when it is immersed in water. The eggs were allowed to develop in water at $12 \pm 1^\circ\text{C}$ until the sampling for the analysis was received. All the developmental stages were prepared from the eggs of the same female.

The egg membrane of the activated salmon egg is more opaque than that of the unactivated egg (Yamamoto, 1957). Owing to this membrane's opaqueness, the developmental stage of the egg cannot be determined in the fresh state. Therefore, 10 eggs were fixed in Bouin's fluid at the same time with the sampling for the quantitative analysis of nucleic acids, and the embryos dissected out of the egg membrane were examined. The developmental stage of the embryos was determined according to the description by Mahon and Hoar (1956). When necessary, the embryos were sectioned by the paraffin method and stained with Delafield's hematoxylin. The cleavage rates of the eggs determined at the 8-16 cell stage (15.5 hours after immersion in water) were always over 80%. Table 1 shows the cleavage rates and the developmental stages at the time of sampling for this analysis. The developmental stages reached after a definite period were nearly the same in all females.

The concentrations of spermatozoa in the semen were determined using the hemacytometer. The concentrations varied with different males and are shown in table 2.

Table 2. Concentration of spermatozoa in semens of different males

Date of collection of male	Oct. 17	Oct. 22	Oct. 29	Nov. 5	Nov. 12	Nov. 19
Number of spermatozoa per ml semen ($\times 10^6$)	192.1	235.8	143.2	143.7	108.9	177.9

Analytical procedure

For the quantitative analysis of nucleic acids, 50 eggs of each developmental stage were fixed in 80% ethanol at the time indicated in table 1 (1°C , overnight). Then the eggs were thoroughly crushed at 1°C without removing the egg membrane. In the case of spermatozoa, 0.1 ml semen was used for analysis.

The extraction of nucleic acids was first attempted according to the method of Schmidt-Thannhauser-Schneider (S-T-S) (Mizuno, 1969). This method was successfully applied for determining both the RNA and DNA in the loach egg (Timofeeva and Kaviani, 1964). However, when the same method was applied for the dog salmon egg, no satisfactory extraction of nucleic acids could be obtained. When the complete absorption spectrum from 200 to 300 nm was read, the extracts

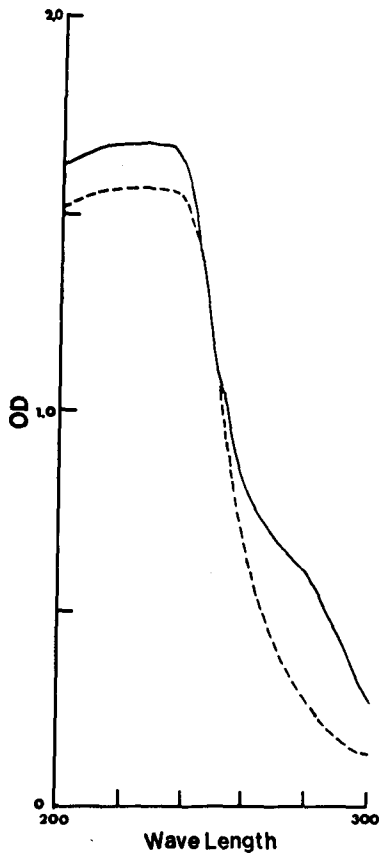


Fig. 1

Fig. 1. The UV absorption spectra of RNA (----) and DNA (—) extracts from unactivated salmon eggs prepared by S-T-S method.

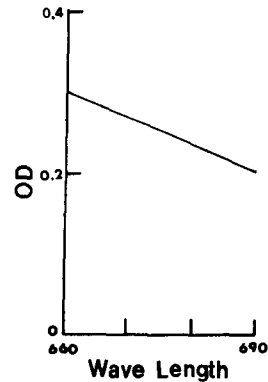


Fig. 2

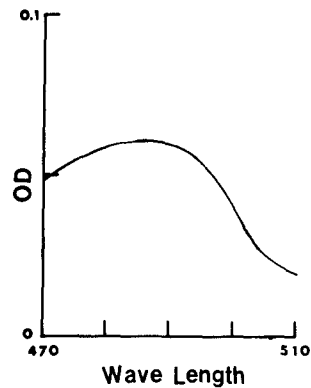


Fig. 3

Fig. 2. The absorption spectrum of RNA extract from unactivated salmon eggs prepared by S-T-S method and subjected to orcin reaction. No peak of absorption curve is observed.

Fig. 3. The absorption spectrum of DNA extract from unactivated salmon eggs prepared by S-T-S method and subjected to indole reaction.

showed no characteristic curve for nucleic acids (Fig. 1). Furthermore, the extract did not show a maximum absorbance at 675 nm after Ceriotti's orcin reaction for pentose (Fig. 2) (Mizuno, 1969). When the extract was treated with Keck's method for deoxyribose (Mizuno, 1969), a maximum absorbance of the sample was found at 490 nm (Fig. 3) but the absorbance values were very unstable in repeated experiments. Therefore, the S-T-S method was not applicable for the extraction

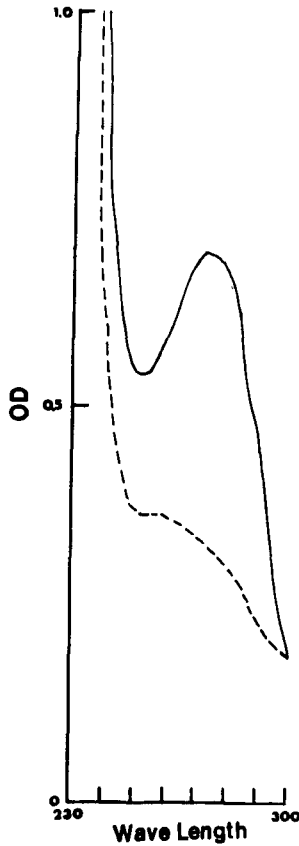


Fig. 4

Fig. 4. The UV absorption spectra of RNA (----) and DNA (—) extracts from unactivated salmon eggs prepared by the method of Ogur and Rosen.

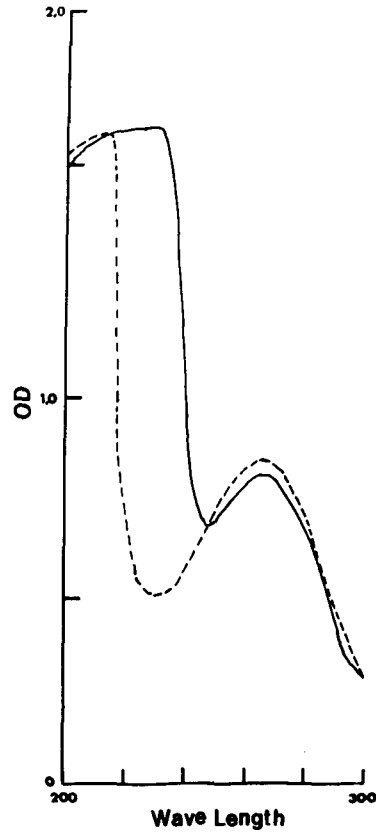


Fig. 5

Fig. 5. The UV absorption spectra of total nucleic acid extracts from unactivated eggs (—) and spermatozoa (----) prepared by the method of Schneider-PCA.

of nucleic acids from the dog salmon eggs.

The differential extraction of RNA and DNA using the Ogur and Rosen method (Mizuno, 1969) was also attempted. In the extracts prepared by this method, the maximum absorbance of DNA fraction was not found at 260 nm but existed near 280 nm (Fig. 4). Therefore, the very intense UV absorption by the degradation products of the egg protein made the measuring of the weak UV absorption of DNA at 260 nm impossible.

On the other hand, when the extraction of total nucleic acids (RNA+DNA) using the Schneider's method, where 16% perchloric acid (PCA) was used instead

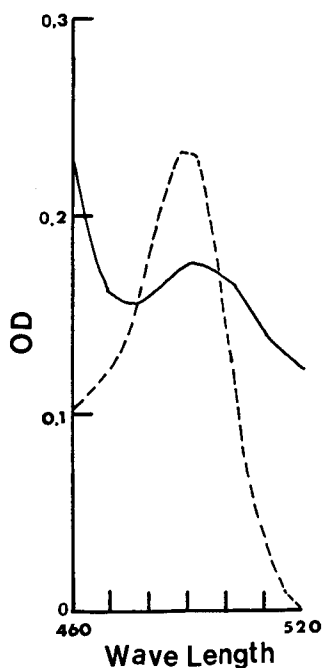


Fig. 6

Fig. 6. The absorption spectra of total nucleic acid extracts from unactivated eggs (—) and spermatozoa (----) prepared by the method of Schneider-PCA and subjected to indole reaction.

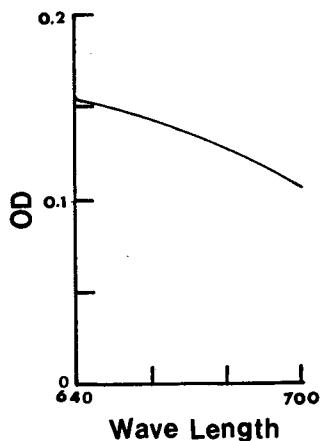


Fig. 7

Fig. 7. The absorption spectrum of total nucleic acid extract from unactivated salmon eggs prepared by the method of Schneider-PCA and subjected to orcin reaction. Note that there is no peak of absorption curve.

of 5% trichloroacetic acid, was attempted (Mizuno, 1969), the maximum absorbance of the extracts was always found at 266 nm (Fig. 5). Furthermore, such prepared extracts showed an absorption curve with a peak at 490 nm after the indole reaction (Fig. 6). In the case of the orcin reaction, however, the absorption curve from 640 to 700 nm had no peak, as seen in Figure 7. In spite of this deficiency, the extraction of total nucleic acids was performed by the Schneider-PCA method.

The dog salmon egg was considerably rich in lipids, and their insufficient removal made quantitative determination results inconstant. Thus, the crushed eggs were treated first with acetone in a glass centrifuge tube. The treatment continued for more than 12 hours at 1°C, during which acetone was renewed three times. Secondly, the lipids were removed with three changes of an ethanol-ether (1:1, v/v) mixture for a total of 40 minutes. The absorption curves of the ethanol-ether extracts are shown in Figure 8. Judging from this figure, the ethanol-ether

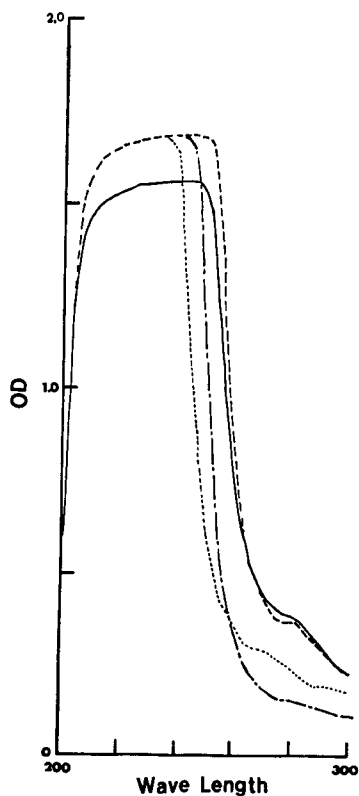


Fig. 8

Fig. 8. The UV absorption spectra of ethanol-ether extracts from unactivated salmon eggs. Extractions were repeated four times at 50°C for 15 min: —, first; ----, second; - - - -, third; ····, fourth extracts.

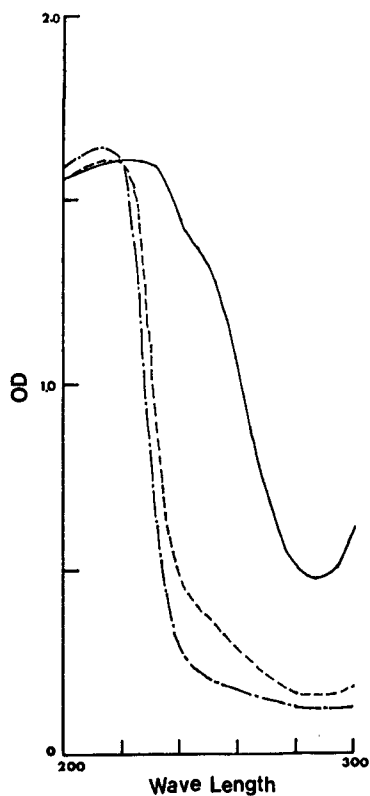


Fig. 9

Fig. 9. The UV absorption spectra of 3% PCA-soluble fractions of unactivated salmon eggs. Extractions were repeated three times at 1°C for 15 min: —, first; ----, second; - - - -, third extracts.

treatment should reduce further changes, because more extraction causes a degradation of protein. The acid-soluble compounds were removed by treating the crushed eggs with three changes of 3% PCA for a total of 45 minutes (at 1°C). The absorption curves of 3% PCA extracts are shown in Figure 9. From this figure, it is clear that three changes of 3% PCA seem to completely remove the acid-soluble compounds. After 3% PCA was discarded, 7.5 ml of 16% PCA was added for the extraction of nucleic acids. The centrifuge tube was allowed to stand exactly 15 minutes at 70°C, and then was cooled. Following centrifugation, the supernatant was collected in a 25 ml volumetric flask. The residue was treated once more with 16% PCA in the same manner, again centrifuged, and the super-

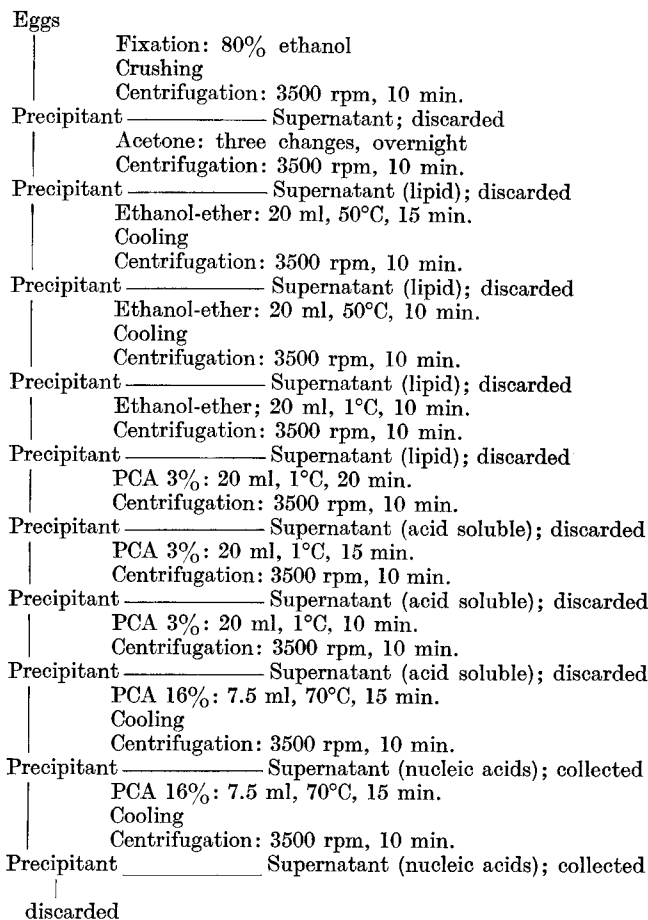


Fig. 10. Scheme of execution for nucleic acid extraction from salmon eggs.

natant was combined with the previous supernatant in the same flask. The scheme of extraction is illustrated in Figure 10. After filling the flask to capacity with 16% PCA, the extract was further centrifuged at 10,000 rpm for 1 hour (0°C) and the supernatant was used as the sample for the determination of the nucleic acid concentration.

In the case of spermatozoa, the extraction of acid-soluble compounds was performed using 2% PCA and that of nucleic acids was carried out in 8% PCA. The procedure was, however, the same as that for the eggs. The final sample of the spermatozoa was diluted 1:10 with the extracting agent.

For the quantitative analysis of DNA, 3 ml of the samples were subjected to Keek's indole reaction (Mizuno, 1969) in the reaction tubes. After shaking with

two changes of amyloacetate, the maximum absorbance was read for the determination of DNA concentration.

As standard, deoxyribonucleic acid from a calf thymus (Sigma Chem.) was used. The substance was dissolved in 16% PCA at 70°C until the solution became water clear. Solutions of adequate DNA concentrations (2–24 μg DNA/ml) were pipetted into the reaction tubes and subjected to the same indole reaction.

For measurements, the complete absorption spectra of each sample from 200 to 300 nm (for total nucleic acids) and from 460 to 520 nm (for DNA) were read using the Hitachi Model 124 recording spectrophotometer. After checking for serious contaminations of interfering substances (Fig. 5), the absorbance values at definite wave length were read using the Hitachi Model 139 spectrophotometer. PCA (16% and 8%), with or without being subjected to the indole reaction, served as a blank for determining the concentration of DNA and total nucleic acids. As previously mentioned, the maximum absorbance of total nucleic acids in the samples prepared by the above procedure was found at 266 nm. The amount of total nucleic acids in the sample was estimated, however, from the UV absorption values at 260 nm using a conversion factor of 0.286 OD_{260} for 10 μg of nucleic acids per milliliter of the sample. The 266/280 ratios of density were 1.15–1.33 in the samples prepared from the eggs and were 1.39–1.45 in those from the spermatozoa. These values indicate an adequate separation of nucleic acids from proteins. In the case of indole reaction, the maximum absorbance at 490 nm was read.

Quantitative analysis

From the UV absorbance value it was calculated that a spermatozoon contains 2.6 pg nucleic acids in average. After the indole reaction, the amount of DNA was 2.7 pg. These results indicate that the amount of nucleic acid (DNA) determined by the indole reaction is slightly higher than that calculated from the UV absorption value.

As has been shown in urodelan eggs by Chen (1960), the amount of total nucleic acids in an unactivated egg varied slightly in different females ranging from 10.2 to 14.4 μg (average 13 μg). Although the diameter of egg was not measured in the present study, the egg size was almost uniform within single female eggs and the larger eggs from a certain fish seemed to contain more nucleic acids than the smaller ones of the other fish. During early embryonic development, however, the pattern of quantitative change in the nucleic acids was the same for the eggs from different females. The average values for six females are indicated in Figure 11. The figure shows that the content of nucleic acids decreased after activation. This decrease continued for at least 40 hours (until mid blastula stage) and usually reached 28% of the original amount. An increase in the total nucleic acids was recognized about 70 hours after activation (late blastula stage) and the nucleic acid content of egg, whose embryo was 2 mm in length, exceeded that found in the unactivated egg.

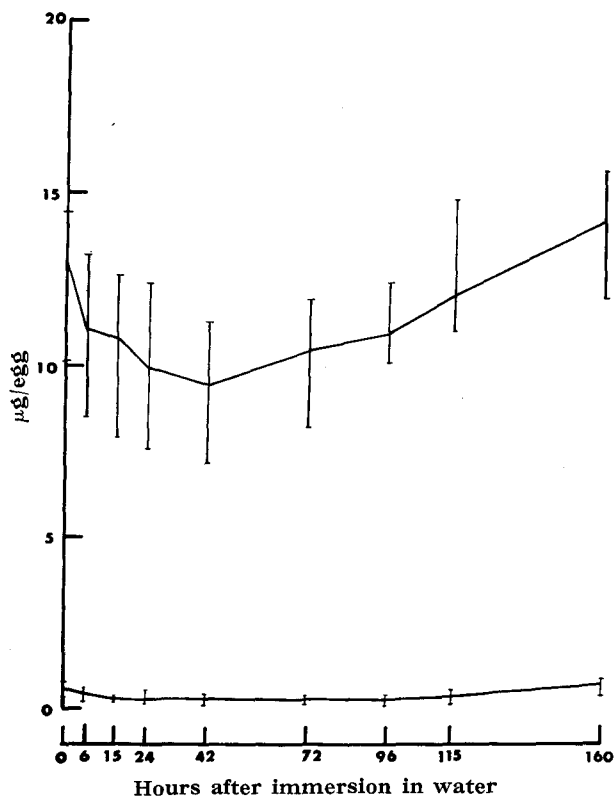


Fig. 11. Quantitative changes of total nucleic acids (upper curve) and DNA (lower curve) during early development of salmon.

The amount of DNA in an unactivated egg also varied in different females ranging from 0.4 to 0.8 μg . As observed in the case of total nucleic acids, the DNA content of the female eggs showed nearly the same quantitative change during the development. Figure 11 shows the average DNA content of the developing eggs. The amount of DNA decreased during the first 24 hours after activation but did not change between 24–96 hours. The increase of DNA content was recognized at 115 hours after activation (germ ring stage) and at the stage of differentiation (160 hours after activation) it was an average of 0.7 μg .

Since the quantitative data obtained by Keck's method is based on a different principle from that of the UV absorption value, a simple subtraction of DNA value obtained in the present study from the amount of total nucleic acids (DNA+RNA) does not give the RNA content of developing eggs. However, as found in the comparison of two methods using the spermatozoa, the difference of the values obtained is very small. Therefore, the amount of RNA was calculated by this

method. It was surmised that the synthesis of RNA in the developing egg becomes active before the increase in the amount of DNA.

Discussion

Few measurements of nucleic acids in fish eggs have been made. Timofeeva and Kaviani (1964) measured the amounts of RNA and DNA in developing loach eggs. They found 0.025–0.035 μg DNA and 2.4–2.6 μg RNA in the unfertilized egg of *Misgurnus fossilis*. Kong *et al.* (1971) showed that a perfect linear relation exists between the amount of DNA and the cube of egg radius in some amphibian species. A larger amount of nucleic acids may be therefore expected in the salmonid eggs than in the loach eggs, since the radius of the salmonid egg is apparently greater than that of the loach egg. In the rainbow trout, Hagenmaier (1969) investigated the quantitative changes of nucleic acids during development in embryos separated from the yolk. Although the exact values of DNA and RNA contents have not been described in this paper, the amounts can be roughly read from his figure; about 2.5 μg DNA and 3.8 μg RNA/cleaving blastodisc. It is known that yolk contains a small quantity of nucleic acids in various animals (Brachet and Quertier, 1963; Brachet and Ficq, 1965; Emanuelsson, 1969, 1971). Therefore, the nucleic acid content of the rainbow trout egg with yolk may exceed these amounts. Taking the egg size into consideration, the values found in the loach and rainbow trout eggs are of the same order of magnitude as the present result and are smaller than those reported in the hen's eggs (Solomon, 1957). Nishida and Watanabe (1966) performed the quantitative determination of DNA in the growing and developing dog salmon eggs. They prepared DNA samples with the method elaborated by McIntire and Sproull (1957) and found 49.2 μg DNA in the ripe unfertilized egg from a fish captured at the Chitose Hatchery and 83.8 μg DNA in the egg from a fish found at the Otoe Hatchery in Hokkaido. These amounts are about 100 times greater than those obtained in the present study. It is not clear whether the disparity in absolute amounts of DNA indicates a technical problem or not. In this connection it should be emphasized that the procedures of extraction for nucleic acids commonly used in the somatic tissues, such as the methods of S-T-S and Ogur and Rosen, are not applicable in lipid- and protein-rich salmonid eggs.

The mean amount of DNA per dog salmon spermatozoon was found to be 2.6 pg, which compares favorably with the reported values of 2.4 pg for the dog salmon (Nishida and Watanabe, 1966) and 2.36 pg for the loach (Timofeeva and Kaviani, 1964).

In agreement with the findings in some vertebrate and invertebrate eggs (Elson and Chargaff, 1952; Brachet, 1954; Durand, 1955; Solomon, 1957; Haggis, 1964; Schwarz, 1970) a comparison of DNA contents in the unactivated egg and the spermatozoon of the dog salmon shows that there is a cytoplasmic reserve of DNA in the egg. Such reserve has also been suggested in other teleostean eggs (Ohi,

1961; Hagenmaier, 1969) and may be located in both active cytoplasm and yolk as shown in amphibian eggs (Brachet and Ficq, 1965).

During embryonic development, the nucleic acid content of the dog salmon egg first decreased. Similar decreases have also been observed in the development of loach eggs. Timofeeva and Kaviani (1964) reported that about 10% of RNA found in the unfertilized egg of *Misgurnus fossilis* is lost by its breakdown during the cleavage stage. On the other hand, Aitkhozhin *et al.* (1964) observed that the amount of nucleic acids (DNA+RNA) shows no change until the stage of gastrulation in the loach egg. The standard deviation of the values of nucleic acids was not calculated in this study. Therefore, it is not clear whether the decrease observed in the dog salmon egg is significant or not. The excess spermatozoa remained on the surface of the unactivated egg in the present study were very few in number. Since very small amounts of DNA were contained in the spermatozoon, the breakdown of spermatozoa remaining on the egg surface may not explain the above-mentioned decrease of nucleic acid content of dog salmon egg. In the histochemical study of the development of dog salmon egg, Nishida (1959) reported releases of substance(s) related to nucleic acids (Ketoenol substance) from the developing egg. This observation might explain the decrease of nucleic acid content of the egg during the early stages of development.

The determinations of nucleic acid content in the embryos separated from the yolk have been performed in loach and rainbow trout. Aitkhozhin *et al.* (1964) observed that the amount of DNA linearly increases in the blastodisc of loach eggs during the cleavage stage and that the increase in the amount of RNA of the blastodisc is also significant during the cleavage stage. Hagenmaier (1969) reported that the increase of RNA content in the blastodisc of rainbow trout egg is initiated at the 16-64 cell stage and that the augment in the amount of DNA begins at the late morula stage. He also indicated that the nucleic acids located in the yolk region of the unfertilized brown trout egg are actively transported into the embryonic region during the early stage of development. In this study, a significant increase in the nucleic acid content of the egg was not revealed until the 2 mm embryo stage. This fact supports the conclusion drawn from the studies on other teleostean eggs that the nucleic acids required to accomplish early embryonic development have been already reserved within the egg cell during oogenesis.

Summary

1. A method designed for the extraction of nucleic acids from lipid- and protein-rich dog salmon egg was described. It is based on the principle used by Schneider. Values obtained by this method were relatively constant in the salmon egg.

2. The amount of DNA in the dog salmon spermatozoon was 2.6-2.7 pg and that of the unactivated egg was 0.6 μ g. From these data it was concluded that there are large amounts of cytoplasmic reserve in the DNA of the dog salmon egg.

3. The nucleic acid content first decreased during embryonic development. The decrease continued until the mid blastula stage. At the late blastula or early gastrula stage the synthesis of nucleic acids was recognized.

4. The analyzed data indicate that the nucleic acids required to accomplish early embryonic development have been already reserved within the egg cell during oogenesis.

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