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Abstract

The present investigation was carried out to see effects of muscle cornin, an alcoholic fraction of boiling-water extract from rabbit skeletal muscle, on the nucleic acid synthesis in the early development of *Pseudocentrotus depressus*. In this study, the author used the assay method of our own device by which we can estimate the incorporation into whole cell simultaneously that into nucleic acid fraction, with one and the same specimen. The results of the observations are briefly summarized as follows. 1) Cornin accelerated the incorporation of 3H-uridine into whole cell by 10-20 %. 3H-thymine, 3H-thymidine and 3H-uracil all inhibited such incorporation. 2) As to the incorporation into the RNA, it was retarded in the course of phosphorylation at the synthetic stage. 3) In the incorporation into DNA, since the incorporation is inhibited by about 2/3 at the synthetic stage, it seems that the polymerization is inhibited. 4) This inhibition of the DNA synthesis was also substantiated by the autoradiography with tritiated thymidine. Some comments were made on the operation of the nucleic acid synthesis, the specific protein structure during the early development of sea urchin egg, and effects of cornin on these as well as on the other intrinsic substances.

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EFFECT OF CORNIN ON NUCLEIC ACID SYNTHESIS DURING EARLY DEVELOPMENT IN SEA URCHIN EGGS

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Many substances extracted from animal tissues are known to inhibit cell division of normal or cancer cells (DRUCKREY *et al.* (6), NAKAHARA *et al.* (31), PARSHLEY (40), HERBUT, *et al.* (18), SZENT-GYÖRGYI *et al.* (47, 48), and others). However, underlying mechanisms of inhibition or acceleration of the cell division are known only in a few of these extracts, and most substances still remain in the stage of investigation. There are only a few reports describing that the rat liver extract inhibits DNA synthesis of the liver cell (BURDON *et al.* (4), TERAYAMA (55) and the human tissue extract similarly suppresses DNA synthesis of Hela cells (NILSSON *et al.* (35).

NISIDA *et al.* (36, 37), prepared an alcoholic fractionation from boiling extract of tissues, designated it "cornin" and reported that the cornin has showed a strong antimetabolic effect on sea urchin eggs at the early stage of mitotic cycle (19, 54) and on some of experimental tumors (17, 39) and culture cells (23, 39).

Especially, muscle cornin is known to inhibit the early stage of mitosis of sea urchin eggs even at a low concentration of 10^{-8} g/ml (22). KANAOKA (22) has demonstrated by the ^{32}P incorporation experiments that the muscle cornin (to be abbreviated as cornin) inhibits DNA synthesis but not RNA synthesis. There is also a report (25) describing that the cornin inhibits the ^{32}P incorporation into the acid soluble fraction of unfertilized sea urchin eggs, 2-cell stage, 4-cell stage and blastula stage.

In the initial mitotic stage of sea urchin egg both DNA and protein syntheses do occur, but as to RNA synthesis, one school claims to see no synthesis of like m-RNA that is directly involved in protein synthesis up to blastula stage and only a minute amount of the precursor is incorporated selectively, and another school of NAMER and INFANTE (32) recognized the synthesis of DNA-like RNA already at 4-cell stage.

The problem at what stage or step cornin affects the nucleic acid

The main part of this report was read at the 46th Congress of Japan Society of Physiology at Yonago, Japan (1969).

synthesis remains still unsolved.

In order to solve this problem an investigation was carried out to see the effects of cornin on the incorporation of nucleosides and nucleic acid bases, labeled with tritium, into sea urchin egg at the early developmental stage, by the method of our own device which enables us to determine the incorporation into whole cell as well as into nucleic acid fraction using Millipore filter. Concurrently, morphological observations were conducted by radioautography with tritium labeled thymidine.

This communication deals with results of these studies.

MATERIALS AND METHODS

Sea water was always passed through Millipore filter (pore size of 0.45μ) before the use (to be called simply as sea water).

All other procedures were undertaken under as sterile conditions as possible. Eggs of *Pseudocentrotus depressus* were obtained by electric stimulation or by intracoelomic injection of 0.5 M KCl.

The eggs were washed several times with sea water prior to use. The sperm, obtained from excised testes, were placed in a dry petric dish. Just prior to insemination one drop of sperm concentration was added to 10 ml of sea water, and one drop of diluted sperm suspension was added to the eggs. Only those batches of eggs in which 95% of the eggs showed fertilization membranes were used.

After diluting the egg suspension, 0.1 ml of it was mounted under microscope for accurate cell counts.

The egg suspension was prepared as to contain 2,400 eggs per milliliter of the final suspension.

Cornin was extracted from the skeletal muscle of rabbit by the method of KANAOKA (22). The muscle was boiled in water for 10 minutes, the hot water extract was immediately cooled, and fractionated into 70–90% alcohol fraction. Then it was rinsed with ethanol,

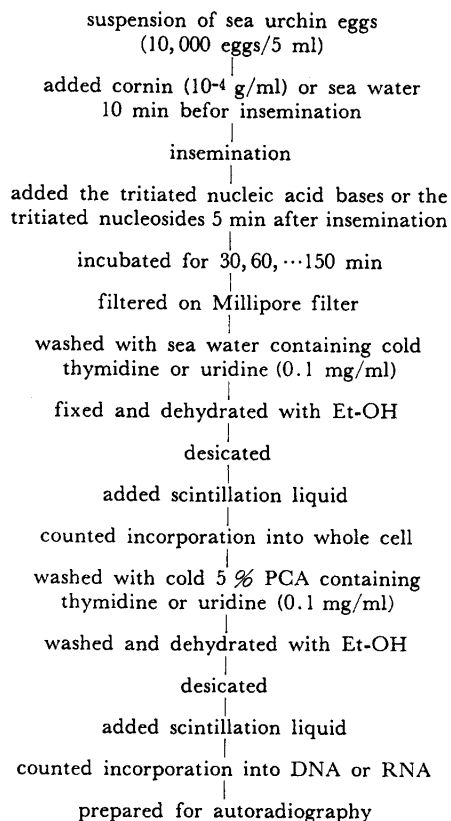


Fig. 1. A simple determination method for nucleic acid synthesis

methanol, acetone and ether. After drying the specimen was stored in a desiccator. The cornin so prepared was dissolved in sea water to the concentration of 6×10^{-4} g/ml before the use and kept in a refrigerator (4°C).

^3H -thymidine (specific activity ; 3.0 C/mM), ^3H -uridine (specific activity ; 5.0 C/mM), ^3H -thymine (specific activity ; 11.2 C/mM) and ^3H -uracil (specific activity ; 16.5 C/mM) were purchased from The Radio Center, Arsham, England. These isotopes were diluted with sea water and stored in the refrigerator.

Taking a pair of 300 ml flasks, 25 ml of the egg suspension was put in each flask, to the control flask 5 ml sea water was added and to the other experimental flask 5 ml of the cornin solution was added, and these were placed in the incubator adjusted to $17.5 \pm 0.5^{\circ}\text{C}$.

The final concentration of cornin in the experimental groups was kept at 1×10^{-4} g/ml. Ten minutes after this pretreatment insemination was done, and 5 minutes later isotope was added. The final concentration of each isotope was adjusted to $0.2 \mu\text{C/ml}$. All these procedures were conducted simultaneously in order to eliminate procedural differences between the control and the experimental groups. The openings of the flasks were covered with aluminium foil to avoid contamination of foreign materials like bacteria. Samples were incubated for 30, 60, 90, 120 and 150 minutes, stirring occasionally.

In order to keep the temperature at $17.5 \pm 0.5^{\circ}\text{C}$ during incubation, two Toyo-incubators were used, connecting by a water pipe to keep one as a heater and the other as a refrigerator, and water bath was kept at the constant temperature by thermostat. At the end of each incubation time the sample was stirred well, and 5 ml of it was strained through Millipore filter (type : HA ; 0.45μ). This was immediately rinsed with 10 ml sea water containing thymidine or uridine (both non-labeled) in the concentration of 0.1 mg/ml.

For the experiments of ^3H -thymidine and ^3H -thymine, non-labeled thymidine solution was used, and for ^3H -uracil and ^3H -uridine, non-labeled uridine solution was used. After the washing, it was dehydrated and fixed with 10 ml of absolute ethanol. The filter on which eggs were adhered was placed in vial and carefully dried under infrared lamps. There was no danger of eggs breaking by these procedures. To the vial was added 15 ml of scintillation liquid, and with Packard tricurve liquid scintillation spectrometer (Model 3003) the incorporation into the whole cell was first estimated. The composition of scintillation liquid was P. P. O ; 5.0 g, P. O. P. O. P. ; 0.05 g in 1,000 ml of toluene. The estimation was done for 5 minutes, which was repeated 3 times, and estimated values were confirmed as valid or invalid by the efficiency of the count to be described later.

Next, the Millipore filter in the vial was desiccated under infrared lamps and washed with 5% cold PCA, (45, 46) which was left standing overnight, exchanging the liquid once, and dehydrated with 80%, 90%, absolute alcohol and dried. Subsequent treatments were identical as in the case with the incorporation into whole cell, and estimates were made of the incorporation into acid insoluble fraction.

Counts of incorporation into the whole cell and the nucleic acid fraction

were confirmed either valid or invalid by the tritium quench curve efficiency of over 30 %. (quench curve of tritium is omitted (11).

Radioautography

The eggs collected at each incubation time in the foregoing incorporation experiments were fixed with acetic acid-alcohol solution (1 : 3, v/v), dehydrated, embedded in paraffin, sectioned to pieces of 5μ in thickness, dipped into the autographic emulsion (NR-M2) of Sakura, exposed for 2 weeks at 4°C , and the photographic development was done.

The first half of the present experiments was conducted at the Marine Laboratory, Okayama University, located at Shibukawa Sea shore of Seto Inland Sea.

Sea urchin eggs used were produce of the northwestern coast of Honshu (San-in coast) and the southwestern coast (Seto Inland Sea coast).

RESULTS

Retarding effects of cornin on early cleavage of sea urchin

When cornin in the concentration of 1×10^{-4} g/ml was made to act on *Pseudocentrotus depressus* being incubated at $17.5 \pm 0.5^{\circ}\text{C}$, both the first and the second cleavage were retarded as showing in Fig 2. At the concentration of 10^{-3} g/ml the cleavage was completely inhibited.

The start of cleavage at the concentration of 1×10^{-4} g/ml was delayed 10 odd minutes, and the termination of the second cleavage was retarded 20 odd minutes.

Cleavage at each period of estimating the incorporation was as follows :

periods

30 minutes. There appeared streak in the egg

of the control group untreated with cornin.

60 minutes. Experimental group treated with cornin began to show streak, but the control was in the state immediately before the start of cleavage.

90 minutes. In the control the first cleavage had completed but in the experimental group the cleavage was in progress.

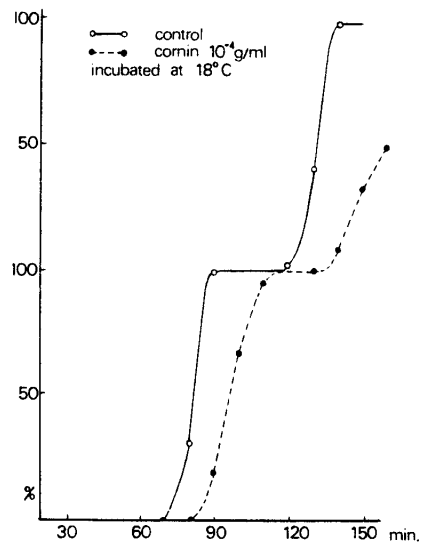


Fig. 2 Retarding effect of muscle cornin on the first and second cleavage of *Pseudocentrotus progressus*. Ordinate shows percentage of 2-cell and 4-cell stage. Abscissa shows time after fertilization (min).

- 120 minutes. Egg of the control started its second cleavage while the experimental one showed streak.
- 150 minutes. In the control the second cleavage was completed but in the experimental one half of the cleavage was finished.

Incorporation into whole cell

These counts were taken with the aim to see how much of 0.2 $\mu\text{C}/\text{ml}$ of nucleic acid bases or nucleosides would be incorporated into the whole sea urchin egg and to determine how the cornin would affect the incorporation of each labeled nucleic acid base or nucleoside.

With exception of ^3H -uridine group which accelerated the incorporation, all the other experimental groups inhibited the incorporation as compared with the control groups.

Tables give the average values of at least several individual counts, and Figs. 3—6 represent the experimental values closest to corresponding average values. Experimental values in each experiment show only slight deviations, their error falling within 5 % range. (Tables 1—4)

Table 1. Incorporation of ^3H -thymine into whole cell and DNA

Incubation time in min	whole cell			DNA		
	control	experiment	per cent	control	experiment	per cent
30	675 cpm	271 cpm	41 %	8 cpm	3 cpm	—
60	1,570	935	60	8	9	—
90	3,080	1,190	40	7	4	—
120	3,815	2,310	61	7	7	—
150	5,650	3,150	56	13	9	—

Table 2. Incorporation of ^3H -thymidine into whole cell and DNA

Incubation time in min	whole cell			DNA		
	control	experiment	per cent	control	experiment	per cent
30	3,750 cpm	2,650 cpm	71 %	52 cpm	32 cpm	63 %
60	9,600	7,800	80	142	91	64
90	14,000	13,300	94	230	180	78
120	19,100	17,300	88	352	201	79
150	21,300	21,300	100	451	350	78

Table 3. Incorporation of ^3H -uracil into whole cell and RNA

Incubation time in min	whole cell			RNA		
	control	experiment	per cent	control	experiment	per cent
30	1,570 cpm	784 cpm	50 %	42 cpm	27 cpm	64 %
60	5,850	2,410	41	47	28	60
90	8,880	5,360	60	54	44	81
120	10,100	5,450	54	75	52	70
150	12,500	7,940	63	86	79	92

Table 4. Incorporation of ^3H -uridine into whole cell and RNA

Incubation time in min	whole cell			RNA		
	control	experiment	per cent	control	experiment	per cent
30	5,930 cpm	6,700 cpm	112 %	37 cpm	24 cpm	65 %
60	14,130	16,800	119	46	34	73
90	24,900	26,800	108	90	60	67
120	31,400	35,900	114	124	112	90
150	41,600	44,600	107	178	132	74

Uracil

The incorporation of uracil increased gradually irrespective of the stage of egg division. Even its incorporation into experimental groups was increased at the rates of 40 to 60 %.

This fact seems to indicate that uracil permeates into the egg cell through the cell membrane irrespective of mitotic stage (Fig. 3).

Uridine

As is obvious from Fig. 4, the incorporation of uridine in the cornin treated groups at every incubation time was 10—20 % greater than that in the untreated controls. The absolute amount of uridine incorporation is greatest among the four kinds. In the control groups the uridine incorporation decreased at 90—120 minutes of incubation, whereas that in the experimental groups kept on increasing.

Thymine

With thymine the incorporation being less, it was suppressed to half in the cornin treated groups as compared with that of the controls. Though it is not certain, the rate of incorporation in controls was somewhat high at the periods of 60—90 minutes and 120—150 minutes corresponding to synthetic periods, but that of experimental groups was low. (Fig. 5)

Thymidine

During the period of 0—30 minutes of incubation the incorporation in

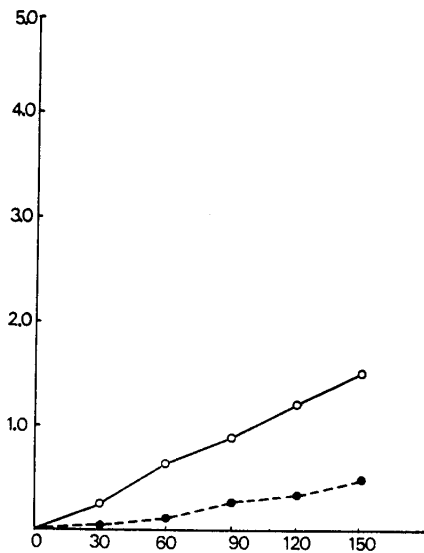


Fig. 3 Incorporation of ³H-uracil

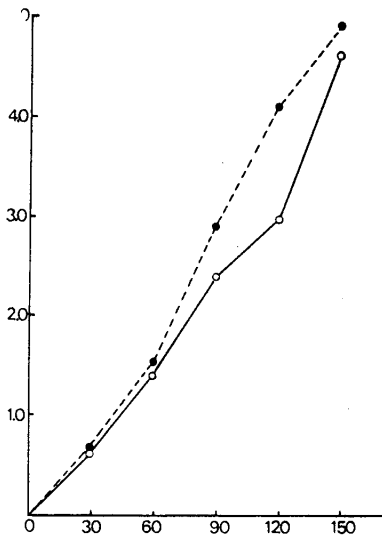


Fig. 4 Incorporation of ³H-uridine

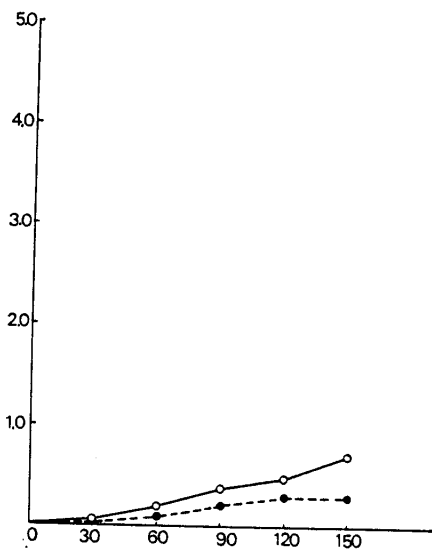


Fig. 5 Incorporation of ³H-thymine

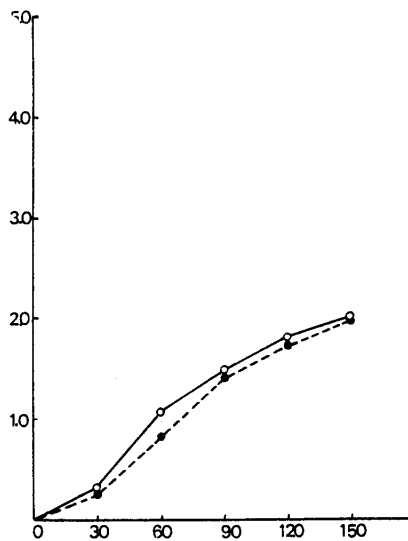


Fig. 6 Incorporation of ³H-thymidine

Fig. 3,-Fig. 6 Effects of cornin on incorporation of tritiated nucleic acid bases and nucleosides into whole cell during early cleavage of sea urchin egg. (○.....○), control. (●.....●), experiment. Embryos were allowed to grow at $17.5 \pm 0.5^\circ\text{C}$ in filtered sea water (control) or in cornin containing sea water (experiment : at a final concentration of 10^{-4} g/ml). Pretreatment of egg, insemination and addition of isotope were done as described in Fig. 1. Ordinate : radioactivity (10^4 cpm/ 10^4 eggs) incorporated during the incubation time exposure (average of counting for 5 min), Abscissa : incubation time in min.

the cornin treated groups was suppressed to 70 % of that in the non-treated controls, and the rate of incorporation approached that of non-treated controls as the incubation time elapsed. This seems to suggest that cornin affects the incorporation only at the beginning, and if given a sufficient time, cornin has little effect on the incorporation into the whole cell. (Fig. 6)

Incorporation into acid insoluble fraction

In the case, among the nucleic acid precursors incorporated into the whole cell the incorporation of those fractions that were insoluble in 5 % PCA was determined. As a result it was found that the incorporation of nucleic acid bases was less while it was considerable with nucleosides. The results in Tables illustrate the average count of many cases, and those in Figs. 7—11 represent the also individual cases. Although the amounts incorporated differed, there could be observed a slight increase of the incorporation in controls at the periods of 0—30 and 90—120 minutes, and in every case the incorporation during these periods was greater than in the cornin treated groups.

Uracil

The amount of incorporation was not so remarkable but in both controls and experimental groups there could be seen a slight acceleration at 0—30 minutes and 90—120 minutes, during the periods corresponding the synthetic period. The synthetic periods in experimental groups were delayed as compared with that of controls and also the incorporation in test groups was suppressed. (Fig. 7)

Uridine

At 30—60 minutes, the resting or interphase, their corporation was less and in controls it increased at 60—90 minutes period while it increased at 90—120 minutes period in test groups. However, in the period between 60 minutes and 120 minutes the total incorporation in controls amounted to 78 cpm and that in test groups 76 cpm, both being about same. This may be interpreted as due to the retardation of synthetic period rather than the inhibitory effect of cornin on nucleic acid synthesis. (Fig. 8)

Thymine

Both control groups and experimental groups incorporated few or none. It seems, the incorporation of ³H-thymine into whole cell is acid soluble. (Fig. 9)

Thymidine

Thymidine gave the most specific results. Many of the test groups showed a general tendency as illustrated in Fig. 10. Namely, the cornin

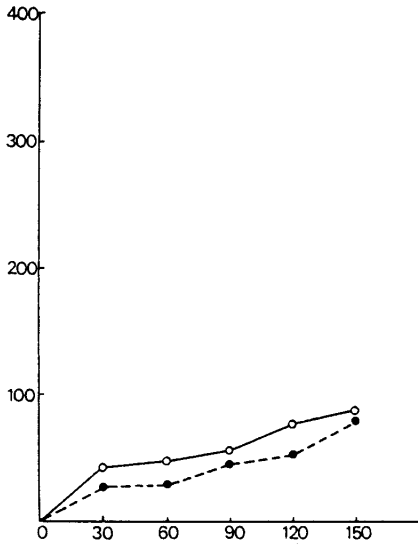


Fig. 7 Incorporation of ³H-uracil

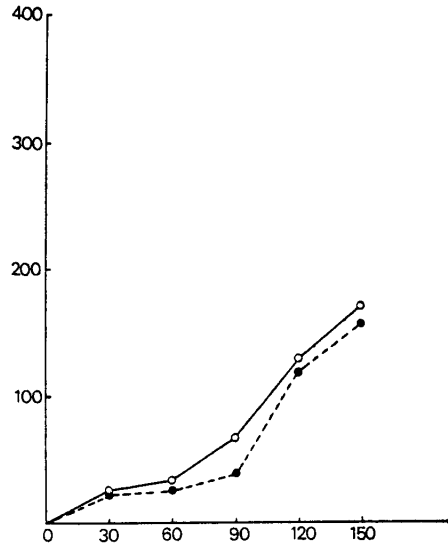


Fig. 8 Incorporation of ³H-uridine

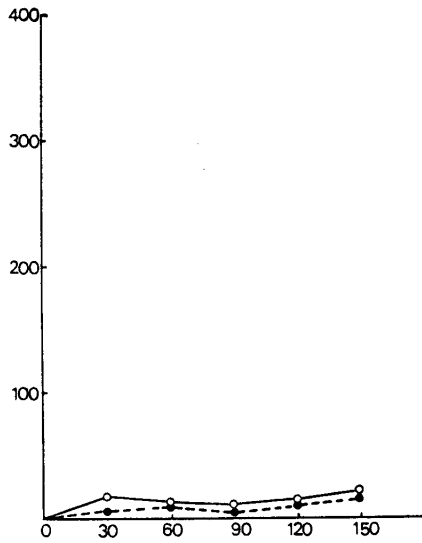


Fig. 9 Incorporation of ³H-thymine

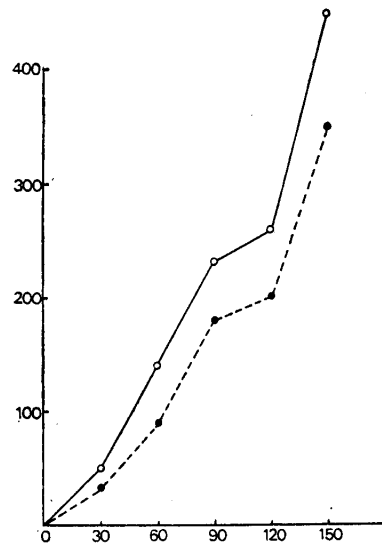


Fig. 10 Incorporation of ³H-thymidine

Fig. 7-Fig. 11 Effects of cornin on incorporation of tritiated nucleic acid bases and nucleosides into acid insoluble fraction during early cleavage of sea urchin egg. (○····○), control. (●····●), experiment. Embryos were allowed to grow at $17.5 \pm 0.5^\circ\text{C}$ in filtered sea water (control) or in cornin-containing sea water (experiment: at final concentration of 10^{-4} g/ml). Pre-reatment of egg, insemination and addition of isotope were done as described in Fig. 1. Ordinate: radioactivity (cpm/ 10^4 eggs) incorporated during the incubation time exposure (average of counting for 5 min). Abscissa: incubation time in min.

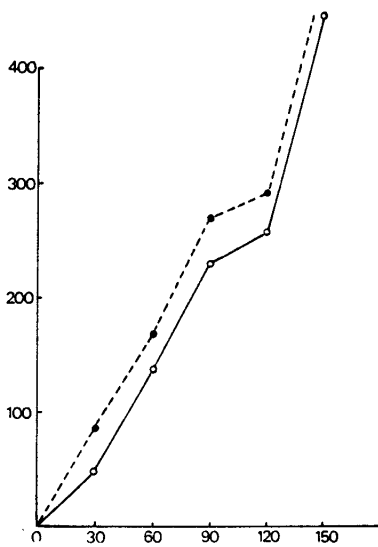


Fig. 11 Incorporation of ^3H -thymidine (few cases)

treated groups had their inhibited incorporation.

In most of these cases the incorporation was inhibited at the synthetic periods of 30—60 minutes and 120—150 minutes down to about 2/3 that of controls. Differences between the test groups and the controls became cumulative at each step of the mitotic cycle. However, in few test groups despite there was not any faults in experimental conditions, cornin effected as to accelerate the incorporation.

In the few cases whose incorporation was accelerated, the incorporation was larger to the same degree in each stage of mitosis and their synthetic periods coincided with those of the majority of cases. (Fig. 11)

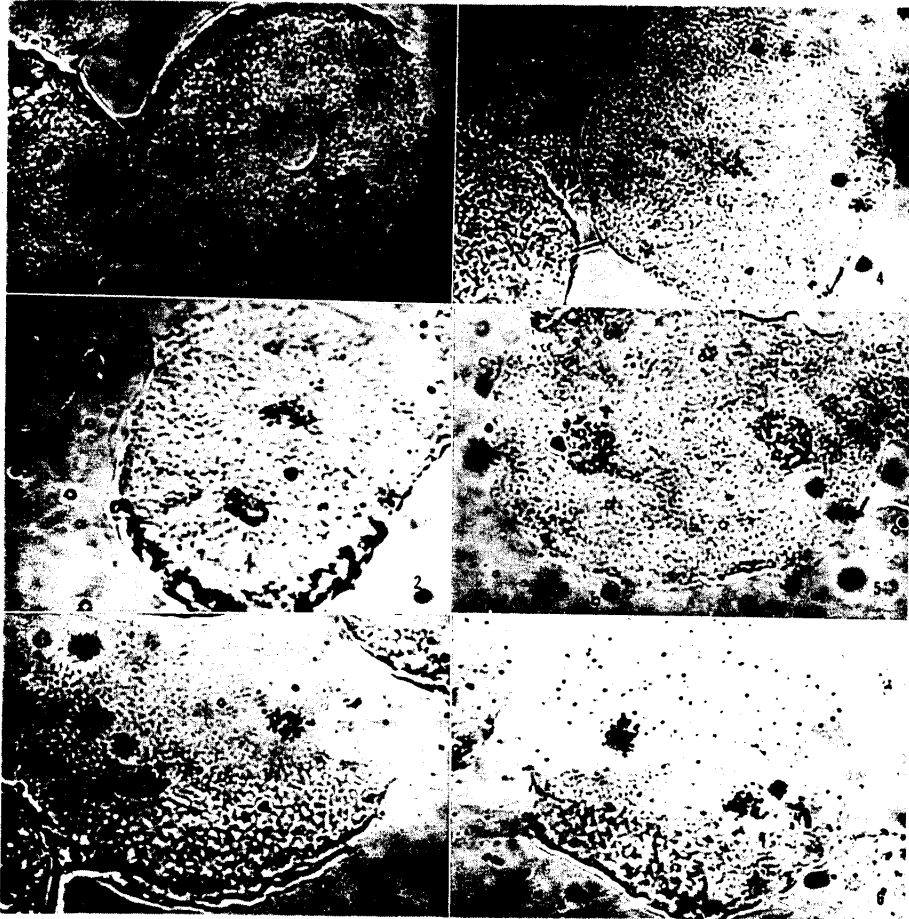
Radioautography of tritiated thymidine

DNA synthesis at the early stage of sea urchin mitosis had been found to occur cumulatively in the nucleus. In the observation of cornin effects the radioautography of ^3H -thymidine gave the findings that morphologically supported the results of the incorporation experiments. Namely, at the 30 minutes incubation the number of grains was less both in controls and test groups, showing no clear cut differences, but as the incubation time elapses, the grains increased in number and they had become aggregated into the nucleus. (Photos. 1—6)

DISCUSSION

The method used in the present experiments to determine incorporation of labeled nucleic acid bases and nucleosides into free cells, using Millipore filter set, has the following advantage over the generally used routine methods.

- 1) There is no danger of cells adhering on to instruments so that complete incorporation counts can be taken.
- 2) It is possible to determine the incorporation into whole cell simultaneously with that into acid insoluble fraction, and also radioautographic studies with one and the same specimen.



Radioautographic demonstration of incorporation of ^3H -thymidine into sea urchin embryos. Treatments were done as described in method.

Experiment

Photo 1 Embryos were incubated in cornin-containing sea water for 60 min.

Photo 2 Embryos were incubated in cornin-containing sea water for 90 min.

Photo 3 Embryos were incubated in cornin-containing sea water for 120 min.

Control :

Photo 4 Embryos were incubated in sea water for 60 min.

Photo 5 Embryos were incubated in sea water for 90 min.

Photo 6 Embryos were incubated in sea water for 120 min.

Effects of cornin on DNA synthesis

The fertilized sea urchin egg contains a considerable amount of pooled nucleosides or nucleotides low molecular form in its cytoplasm. However, it is widely known that DNA synthesis occurs in sea urchin egg from early

stage of the development and that thymidine phosphate, the gene of DNA precursors is incorporated in the process of the polymerization of these precursors.

Concerning the relationship of DNA synthesis to the cell division it is said that the synthesis starts just before the first cleavage commences, and it is completed immediately after termination of the egg cleavage.

According to, NAGANO and MANO (30) who studied the correlation between ^{32}P , thymidine, thymidine kinase, and thymidilate kinase on one hand and egg cleavage on the other, after the second cleavage there is an increase of enzymic activity in between the cleavage, and the incorporation of ^{32}P occurs immediately after it, and the egg cleavage takes place in between the DNA synthesis and the increase in ^{32}P incorporation.

These results coincide well with the present results in that the rate of the incorporation of ^3H -thymidine is high during the 30 minutes period before and after the egg cleavage.

Therefore, it may be said that cornin does not affect the DNA synthesis of egg cleavage stage, but it operates on the process of phosphorylation. In other words, it operates during the process of thymidine \rightarrow TdMP \rightarrow TdDP \rightarrow TdTP \rightarrow DNA or during the polymerization period. The fact that cornin in the meantime inhibits about 2/3 of DNA synthesis coincides well with the finding of KANAOKA (22) and KOSHIMUNE (25). However, in the case with mitomycin-C used in similar manner as cornin, it was only effective at the concentration of 10^{-4} g/ml \sim 10^{-5} g/ml, while on the contrary, chromomycin (Toyomycin) which is an inhibitory agent of DNA synthesis showed stronger inhibitory effect on the cell division. (both unpublished data.)

Judging from these data, it can be understood that the effect of cornin is not simply inhibition of nucleic acid synthesis such as DNA synthesis. Since the ratio of incorporation into whole cell to that into acid insoluble fraction is less in the cornin treated groups, there is a possibility of inhibiting effect of cornin at the polymerization stage, but this might not be identical with the case of mitomycin-C which binds firmly two chains of DNA by itself to the latter.

TERAYAMA (55), and OTSUKA and TERAYAMA (38) also pointed out that the extract of normal rat liver inhibits the nucleic acid synthesis at the cell cleavage stage and state that biochemically it does not inhibit at the polymerization stage but in the course of phosphorylation. In contrast, BURDON *et al.* (4) claim that rat liver extract shows inhibitory effect on DNA polymerase and this is probably due to the action of DNase on DNA.

Even considering the report of NILSSON and PHILIPSSON (35) in which

they state that human tissue extract inhibits DNA synthesis of Hela cell and this inhibitory effect operates prior to protein synthesis, it seems to be the fact that tissue extract has inhibitory effect on DNA synthesis and also inhibitory effect on mitosis.

Effect of cornin on RNA synthesis

As summarized by NEMER and INFANTE (32) there are many investigators who contend that mature sea urchin egg has functional ribosome and m-RNA which are synthesized in the process of egg formation and it requires protein synthesis up to quite advanced stage but does not need RNA synthesis. (NEMER and BRAD (34), and others). ESPER likewise reported that young oocytes already possess a considerable amount of RNA making it unnecessary to synthesize RNA in early cleavage, and RNA synthesis becomes vigorous only after blastula stage (8). Somewhat similar results have obtained with amphibian egg (3), and such a tendency seems to be common to the early developing. It is reported that ribosome of mature egg is the common constituent as polysome, which requires no synthesis in the nucleus and the time when the amount of newly synthesized ribosome becomes greater than that of egg ribosome which has been in the unfertilized egg prior to fertilization is 40 hours after fertilization in *Strongylocentrotus purpuratus* (32). However, there is a report (28) stating that some of unfertilized egg ribosomes are transformed to polysomes soon after fertilization and these polysomes play the role of amino acid incorporation. Against these reports contending that after fertilization of the egg, RNA synthesis does not occur, or it is unnecessary, there are dissenting reports as follows.

WHITELEY *et al.* (42) report that even an unfertilized egg already incorporates RNA precursors through in a minimal amount, and after fertilization it increases rapidly and its synthesis can be inhibited by 2—4 dinitrophenol. Hence they contend that in the initial stage of sea urchin cleavage nucleoside on the cell surface seems to be converted to phosphate.

NEMER and INFANTE (33) have observed the hybridization of DNA-like RNA already at the 4-cell stage, and this RNA, they consider, is derived from polysome at gel-interphase. they have studied the replenishment of this RNA to DNA. As a result they have divided this newly synthesized DNA-like RNA into light polysomes and particles distinct from the cytoplasmic RNA or maternal messenger RNA and heavy polyribosomes of the young embryo.

A great number of reports upholding RNA synthesis in the early stage of sea urchin egg cleavage, also present discrepancy to changes in RNA synthesis by fertilization itself as in the contentions denying RNA synthesis.

There is also a report that in unfertilized egg nuclear apparatus and chromosomes are synthesizing RNA, but when the nuclear membrane is broken by fertilization, this synthesis ceases and this synthetic activity remains inert throughout 2 to 4-cell stages, and the RNA present in between prometaphase and metaphase has been synthesized before the entrance of sperm (5). Conversely, BRACHET *et al.* (2) state that even unfertilized egg incorporates RNA precursor though only in a minimal quantity but this incorporation is limited to a certain extent. Nevertheless, they have observed a rapid increase in such incorporation after fertilization. They have pointed out that, since 2-4 dinitrophenol inhibits the incorporation of uridine, the fertilization is involved in phosphorylation, and consider that the fertilization is accompanied by changes in phosphate donors or enzymic changes. GUIDICE and HÖRSTADIUS (16) are of the opinion that, as actinomycin-D shows no inhibitory effect on the embryo up to 16-cell stage, the embryo up to that stage has animal potentiality in itself regardless of nucleic acid synthesis. There are reports as by FICQ (10) in that precursor is incorporated into a certain portion of the nucleus, and the other (12) that depending upon the stage of fertilization even at 4-cell stage there can be observed ^{32}P incorporation into the terminal pCpCpA of transfer-RNA. In other words, there are considerable differences in the results of investigations on the RNA synthesis during early development of sea urchin eggs, depending upon the subjects and methods of assays used for the period covering fertilization and blastula stage.

Judging from these reports, it can be safely said that in the early stage of sea urchin egg cleavage there is no need of synthesis of m-RNA as the information carrier for the protein synthesis required for the egg cleavage itself, but there certainly occur the incorporation of precursors in minimal amount and changes in ribosome, particularly RNA synthesis of low molecular form to undergo phosphorylation.

Recently, SINGH (49) states that at the stage soon after fertilization the incorporation of adenine into nucleic RNA is slight, and its incorporation into high molecular RNA occurs at least several hours after the start of egg cleavage. Therefore, it is only natural that the amounts of incorporation of uracil and uridine in the present experiments are minimal.

The fact that the retardation of incorporation by effect of cornin appears in the form of retardation of the synthesis, may be more rational to interpret, even it has some effect on m-RNA, as not inhibition of the synthesis but some effect upon the operation itself of m-RNA.

Previously, KANAOKA compared the fraction III of cornin with histone and pointed out the following differences (22). Both of these substances

are separated as practically identical fraction on DEAN cellulose column, and even their ultraviolet absorbancy draws about similar curves, and histone in the concentration up to 10^{-5} g/ml inhibits egg cleavage of sea urchin. On the other hand, as to their effects on nucleic acid synthesis arginine rich histone inhibits RNA synthesis, whereas cornin inhibits DNA synthesis, showing difference in their mechanisms. However, in the investigations of ^3H -uridine incorporation conducted for the purpose to study the effects of heterologous (commercial arginine rich histone and calf thymus histone) and homologous histones on the RNA synthesis of asterias, FICQ (9) observed every one of the histones to stimulate metabolism. Supposing that such a phenomenon is not only specific to asterias but it is a characteristic common to oocytes, then the effect of histone on RNA synthesis in the finding of FICQ insulated contradictory to the findings of MIRSKY *et al.* (1), and UTSUMI, MURAKAMI and others (58). As the same time, of the results obtained in the present experiments there was finding clearly contradictory. Perhaps this finding might have some connection to the effect of cornin on the DNA synthesis.

Protein synthesis and other problems

The process of protein synthesis which ensues the nucleic acid synthesis also constitutes the core of cleavage mechanism, and there are many reports on it (20, 24). It is possible to utilize ^{14}C -glucose in unfertilized egg but its incorporation into amino acid synthesis seems to have not been recognized (29). Soon after fertilization incorporation commences, and Gross and FRY (15) state that it increases gradually at the first and the second cleavage. In contrast, in the identical ^{14}C -leucine incorporation experiments SOFER *et al.* (50) have observed that the incorporation of ^{14}C -leucine increased linearly up to prophase, but it kept decreasing through metaphase and anaphase and then again it rose at telophase. MONROY (28) reports that at the 32—64 cell stage when the cell cleavage is vigorous, once the incorporation falls and it rises again. In addition, attempts have been made to replace non-labeled amino acid of sea urchin egg with labeled amino acid in the early development, the results have not disclosed clear cut differences (41). STEPHENS (52) have observed actine-rich protein to be localized in the mitotic apparatus, flagella and cilia. The behaviors of these specific proteins are important because they have a direct role in mitosis. Especially the mitotic apparatus as well as its outer layer cortex have attracted much attention since SWANN and MICHELSON (14, 53). With a special interest in a close relationship between SH group of the mitotic apparatus of sea urchin egg and mitosis, SAKAI *et al.* (44) have made a significant contribution to changes in soluble protein, water soluble

protein and insoluble protein. The most active among the pH 5 proteins that convert SH and SS are said to be ribonucleic proteins as low molecular components.

IKEDA and WATANABE (21) have also obtained similar results in the behaviors of SH group with tetrahymena. Now, it seems very reasonable to see an idea rising that there might be differences in the protein synthesis between the mitotic apparatus and other bulk of cell proteins. MANGAN *et al.* (26) state that the incorporation of amino acid into the mitotic apparatus is three times as that of other regions but such incorporation amounts to only 11% of the total incorporation, and while hinting that such an incorporation may serve a role in the fibrous skeleton of the mitotic apparatus itself, they do not attach much significance to it. RINALDI (43) and WILT (56) have reached similar conclusion in the first cleavage the absence of new protein synthesis would not impede the cell division. One of the aspects that can not be overlooked in relation to the mitotic mechanism is the aspect of high energy phosphorylation.

MIKI (27) has measured the ATP activity of cortical material from fertilization to 2-cell stage and found that the ATP activity reaches its maximum at metaphase and it decreases gradually thereafter. WEISENBERG and TAYLOR (57) have found the ATPase activity is localized in 13 ± 1 S by sucrose gradient centrifugation, which is activated by Mg^{++} and Ca^{++} , and between pH's 7.5 and 9.5 its activity is pH dependent. However, it seems that of those variations in ATPase of each mitotic stage similarly as in the protein synthesis, although ATPase is a component of the mitotic apparatus, it can not be taken as specific one (7). GOLDMAN (13) has likewise observed the mitotic apparatus from morphological aspect and confirmed the presence of RNA and studied changes in ribosome-like protein after treating the mitotic apparatus with RNAase.

These numerous findings on the mitotic apparatus really imply that the mitotic apparatus plays some role in the mitotic mechanism of cleavage, but they have not yet hit the core of the mechanism.

Nevertheless, for purposes to determine whether or not the effect of cornin on the nucleic acid synthesis, which is principally the DNA synthesis, is truly the intrinsic property of cornin effect, it is necessary to determine its effect on the synthesis of specific proteins, especially its effect on the mitotic apparatus and its outer cortex.

SUMMARY

The present investigation was carried out to see effects of muscle

cornin, an alcoholic fraction of boiling-water extract from rabbit skeletal muscle, on the nucleic acid synthesis in the early development of *Pseudo-centrotus depressus*.

In this study, the author used the assay method of our own device by which we can estimate the incorporation into whole cell simultaneously that into nucleic acid fraction, with one and the same specimen.

The results of the observations are briefly summarized as follows.

1) Cornin accelerated the incorporation of ^3H -uridine into whole cell by 10—20 %. ^3H -thymine, ^3H -thymidine and ^3H -uracil all inhibited such incorporation.

2) As to the incorporation into the RNA, it was retarded in the course of phosphorylation at the synthetic stage.

3) In the incorporation into DNA, since the incorporation is inhibited by about 2/3 at the synthetic stage, it seems that the polymerization is inhibited.

4) This inhibition of the DNA synthesis was also substantiated by the autoradiography with tritiated thymidine.

Some comments were made on the operation of the nucleic acid synthesis, the specific protein structure during the early development of sea urchin egg, and effects of cornin on these as well as on the other intrinsic substances.

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