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Abstract

Both the cornea and muscle cornins have no effect at all on oxygen uptake of tissue, and likewise they catalase affect Qctivity not in any way. The corneacornin has an effect to reduce P/O ratio to about one half, but the muscle cornin does not show such an effect. Both comins decrease the incorporation of P^{32} into nucleic acid fraction and DNA synthesis. In the ultracentrifugal analysis of nucleic acids during development of sea urchin eggs, cornins inhibit the polymerization of nucleic acids. In addition, both of these comins depress the incorporation of P^{32} into DNA and ribosome RNA of regenerating rat liver. Both comins inhibit the increase of -SH quantities before the cleavage of sea urchin eggs.

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ANTIMITOTIC ACTION OF CORNIN AS A BIOLOGICALLY ACTIVE POLYPEPTIDE

II. PHYSIOLOGICAL EFFECTS OF CORNIN ON DIVIDING CELL*

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Previously it was reported that "cornin," a substance extracted from beef cornea and rabbit muscle, has a marked antimitotic activity and that each of the cornin can be separated into three fractions by DEAE—cellulose column, and biochemical properties of each fraction have been discussed.^{11,20,21,22}

The present paper is concerned with antimitotic mechanisms of cornin, that is, a series of experiments were conducted to see the effects of cornin on ADP/O ratio of rat liver mitochondria, oxygen uptake, DNA and RNA syntheses of regenerating rat liver, DNA and RNA syntheses of sea urchin eggs, and changes in the quantities of incorporation of P³² into nucleic acids and of cell division and —SH group.

MATERIALS AND METHODS

The extraction of cornea and muscle cornins was performed with the methods described in the previous papers.^{20,22} The cornin used in the present experiment was in a crude form obtained by the alcoholic fractionation alone. The materials used for determining its effects on oxygen uptake were normal and regenerating livers of frog and rat, and the measurements of oxygen uptake were taken by Warburg manometer with glucose and succinate as substrate, using slices, homogenate of liver tissue and liver mitochondria. In order to find out whether or not cornin possesses a toxohormone-like action, 25 mg/100 g of cornin were injected intraperitoneally into mice and rats, and catalase activities of blood and liver were estimated at 240 m μ by BEERS-SIZER's method.¹⁹ Rat liver mitochondria were isolated with 0.25 M sucrose solution containing 5 mM of Tris buffer (PH 7.4)¹². And by means of rotating platinum-electrode with succinate as substrate ADP/O ratio was determined to see the effects of cornin on the P/O ratio.^{9,30,31}

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As for sea urchin the species of *Temnopleurus toreumaticus* in summer (28°—32°C), *Pseudocentrotus depressus* in autumn (14°C) and *Hemicentrotus pulcherrimus* in winter (6°—15°C) were used as in the previous reports.^{20,21,22} The experiments were conducted at the Marine Laboratory of Okayama University during the period from 1962 to 1964. Ovulation and ejaculation were induced by electric stimulation or by an intracoelomic injection of 0.5 M KCl. The shed eggs were then immediately washed twice by decantation and set aside as a stock suspension. Those eggs with the rate of fertilization of over 95 per cent were selected. Cornin was dissolved in sea water to which egg suspension was added as to make the final concentration 10^{-4} g/ml or 10^{-6} g/ml. The unfertilized eggs of sea urchin obtained by KCl injection or electric stimulation were left standing until there were formed 20—30 ml of natural sediment. This sediment was collected and put into two 500 ml. measuring cylinders in equal amount, using one as the control and the other was adjusted to a certain concentration of cornin solution. To both of these 200 to 500 μ C of P^{32} were added immediately and transferred to the petri dishes of 30 cm in diameter, and stirred occasionally. The unfertilized eggs were centrifuged 20 minutes later, and for the fertilized eggs, the eggs were priorly treated with cornin for 20 minutes and then inseminated, and when the control groups showed cleavage in more than 50 per cent of them, it was taken as 2-cell stage and 4-cell stage. In the case of regenerating rat liver, 20 hours after partial hepatectomy 20 mg/100 g of cornin dissolved in Ringer's solution were injected intravenously into rats and either one hour or 47 hours later 300 μ C of P^{32} were injected intraperitoneally and one hour after the injection the animals were sacrificed by decapitation and served for the experiment.

As for the extraction of nucleic acids, in the case of sea urchin, eggs were collected by centrifugation, and after adding 10 volumes of 0.14 M NaCl—0.05 M acetate buffer (pH 6.0) they were homogenized. Then to this homogenate sodium dodecyl sulphate (SDS) was added in such an amount as to make the final concentration 0.5 per cent. Extraction was done in ice-bath, after repeating the extraction and sedimentation three times the incorporation of P^{32} into nucleic acid fractions as well as quantitative analyses of RNA and DNA were done with phloroglucinol and diphenylamine reactions respectively. Further, by means of methylated bovine serum albumin (MBSA) column of SUEOKA *et al.*²⁸ and PHILIPSON'S²⁹ method the fractionation of nucleic acids was conducted. Then the nucleic acids so fractionated were assayed by ultracentrifugal analysis. In the case of regenerating rat liver, to 1 g of liver 10 volumes of 0.14 M NaCl—0.05 M acetate buffer were added and homogenized. Nucleic acids were extracted by SDS—phenol method, the fractionation of nucleic acids by MBSA—column, and quantitative analyses of DNA and RNA were conducted. The quantitative

analyses of nucleic acids were also performed by the method of SCHNEIDER.

The developing eggs were collected by centrifugation at each cleavage stage after fertilization and the sediment mixed with 10 volumes of 1 *M* perchloric acid was homogenized, and after 10 minute centrifugation at 10,000 r. p. m. the quantitative analysis of -SH contained in the supernatant was conducted by polarography.

RESULTS

It has been clarified that there is no difference in the effect of cornin on oxygen uptake as compared with that of control whether the cornin is derived from cornea or muscle and irrespective of the substrates. It has also been found that cornin has no effect on catalase activity and shows no toxohormone-like action. On determining ADP/O ratio by UTSUMI *et al.*^{30,31} and CHAPPEL'S⁹ method, with the cornea-cornin at the concentration of 10^{-5} g/ml it is demonstrated that the P/O ratio falls to about one half as illustrated in Fig. 1. In the case of the muscle cornin, however, even at the concentration of 10^{-4} g/ml the P/O ratio falls

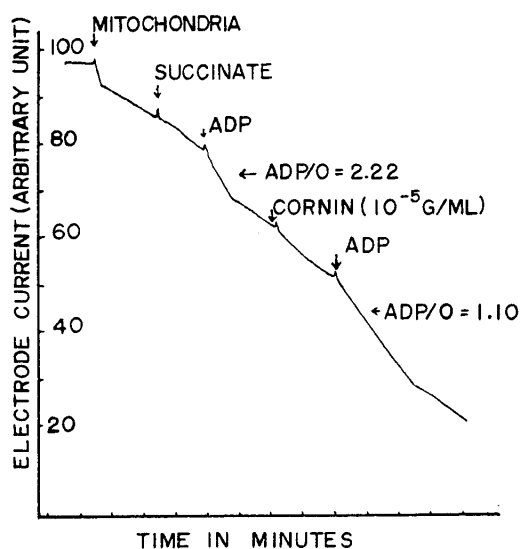


Fig. 1 Effect of Cornin on P:O Ratio of Rat Liver Mitochondria

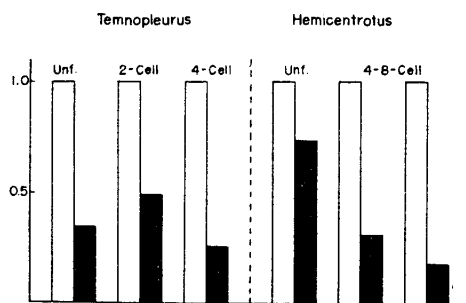


Fig. 2 Inhibitory Effect of Cornin on P^{32} Incorporation into Nucleic Acids of *Temno-pleurus* and *Hemicentrotus* Eggs at Unfertilized, 2-Cell and 4-Cell Stages

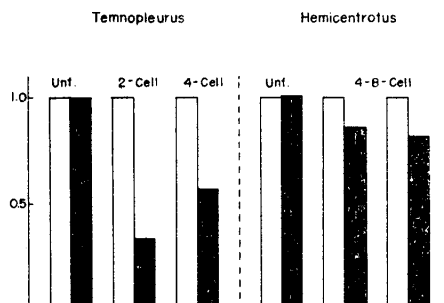


Fig. 3 Inhibitory Effect of Cornin on Deoxyribonucleic Acid Synthesis of *Temno-pleurus* and *Hemicentrotus* eggs

only very slightly.

As for the incorporation of P^{32} into nucleic acids of sea urchin eggs, taking that of control as 1.0, it is inhibited at every cleavage both in the case of *Hemicentrotus* and *Temnopleurus* as shown in Fig. 2. The amounts of DNA do not show any difference in the case of unfertilized eggs as indicated in Fig. 3, but it is less than that of the controls at the 2-cell, 4-cell or 8-cell stage. This decrease is especially prominent in the case with *Temnopleurus*. The RNA contents do

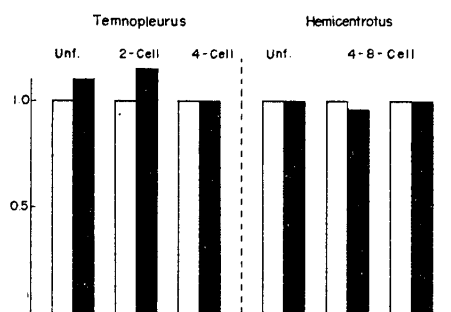


Fig. 4 Effect of Cornin on Ribonucleic Acid Synthesis of *Temnopleurus* and *Hemicentrotus* Eggs

not differ in the case of *Hemicentrotus* as can be seen from Fig. 4, whereas with *Temnopleurus* either there is no difference in the amounts of RNA or it is somewhat greater than that of the control. The results of analyses by the MBSA-column are shown in Fig. 5. Although each pattern of t-RNA, DNA, and r-RNA is distinct, P^{32} is not incorporated in a sufficient amount as to be distinguishable in any one of these patterns. Even with the use of 1 mC

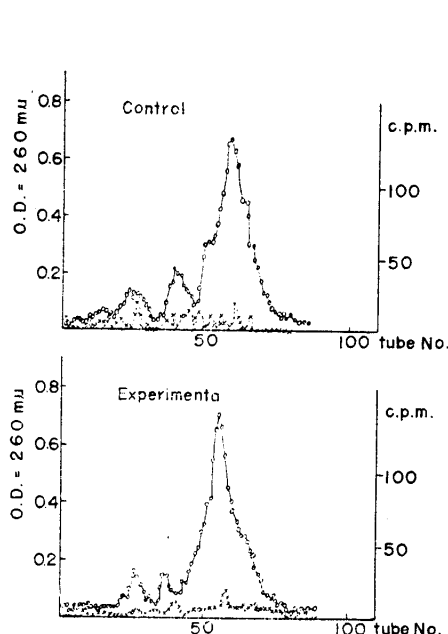


Fig. 5 Fractionation of Nucleic Acids with MBSA-column on *Hemicentrotus* Eggs at 4-Cell Stage, and Incorporation of ^{32}P into Each Fraction

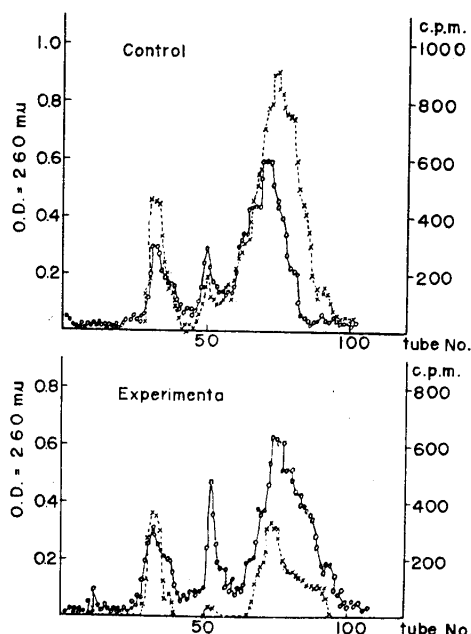


Fig. 6 Fractionation of Nucleic Acids with MBSA-column on Regenerating Rat Liver, and Incorporation of ^{32}P into Each Fraction

—○—, Absorbancy at 260mμ, ... × ..., Radioactivity by counts per min.

of P^{32} similar results are yielded up to the 4-cell stage. In the ultracentrifugal analysis of nucleic acids in *Hemicentrutus* eggs, when treated with 10^{-4} g/ml muscle cornin, there can be recognized no difference of the patterns in unfertilized eggs but there are observed at the 4-cell stage of the control patterns what correspond to those of 19 *S*, 11 *S* and 4 *S*, and in contrast, in the experimental groups treated with cornin the patterns of 17 *S*, 10 *S* and 4 *S* have appeared. On the other hand, in the similar experiment with regenerating rat liver the incorporation of P^{32} into nucleic acids of each pattern is inhibited as shown in Fig. 6, and this inhibitory tendency is especially marked in the incorporation to DNA and ribosome RNA.

With respect to changes of the —SH quantities in the developing stage using *Pseudocentrotus* the increase of the —SH quantity before the first cleavage is markedly delayed as illustrated in Fig. 7.

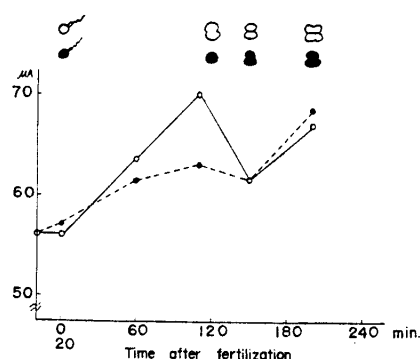


Fig. 7 Change in —SH Groups of PCA-Soluble Protein of *Pseudocentrotus* Eggs During Development

—○— Control ····· Experimenta

DISCUSSION

In previous reports^{20,21} we described about our successful extraction of a substance from beef cornea and rabbit muscle, that showed a marked inhibitory effect on the cell division of sea urchin eggs. When this substance is fractionated by DEAE-cellulose column, three fractions are separated; one of nucleoproteins that shows an antimitotic activity, one with hypoxanthine as its base, and the one of polypeptides that has adenine as its base. Besides these some fractions have been isolated, that are polypeptides by themselves. The fraction isolated from beef cornea and having antimitotic activity is undialysable, whereas most of the fractions isolated from rabbit muscle that have antimitotic activity are dialysable.

According to a recent review by LETTRE,¹⁶ BRACHET,⁷ SWANN,²⁹ BARTH,⁴ BASS,⁵ LEVI-MONTALCINI *et al.*¹⁷ and DUSTIN¹⁰ the numbers of substances that show antimitotic activity are very considerable in kinds, but they can be roughly divided into two large classes, namely, one that acts as an inhibitor of the mitotic apparatus formation and the other that acts as an inhibitor of nucleic acid synthesis. From the findings of the present experiments the antimitotic action of cornin can be represented by the fall in P/O ratio, the inhibition of P^{32} incorpo-

ration into nucleic acids, and the inhibition of DNA synthesis. Even when we mention about the inhibition of mitotic apparatus, just as ZIMMERMAN²⁴ reports, in an isolated mitotic apparatus there is RNA present in the concentration of 5×10^{-7} mg/mitotic apparatus. It is still obscure what physiological significance this RNA has on the formation of mitotic apparatus, but at the present stage of our knowledge where the relationships of DNA—RNA—protein synthesis are being clarified by degrees, it is possible to consider the nucleic acid synthesis and mitotic apparatus formation in the same dimensions.

MIRSKY *et al.*^{1,2} and LEHNERT¹⁵ have reported that arginine-rich histone markedly inhibits RNA synthesis. This substance is a polypeptide that is heat stable and undialysable. There is, however, a possibility of further fractionations by CM—cellulose column and DEAE—cellulose column and it is not clear which fractions show bulk of the antimitotic activity. After fractionating histones by CM—cellulose column and estimating them by polarography, BALZSEK *et al.*⁸ have found a typical protein wave. It is worthy of notice that biochemical properties of fraction III isolated from cornea-cornin by us resemble those of the fraction from histone. Recently we have discovered that the arginine-rich histone is an uncoupler of oxidative phosphorylation in mitochondria.³⁸ Cornea-cornin shows such an action and it seems that the active site of antimitotic activity may be located at such a level. Moreover, the fraction II from cornea-cornin resembles what MENKIN¹⁸ calls a retarding cleavage factor and the fraction II from muscle cornin resembles also the retarding factor reported by WOLFSON³² in the point of permeability and in being a nucleoprotein, although their ultraviolet absorbancy differs from that of cornin.

STEARNS, *et al.*²⁷ BARR,³ and LALLIER¹⁴ have reported that bases and nucleosides such as pyrimidine derivatives, thymidine, adenine, adenosine, guanosine, inosine and uridine show antimitotic effect and inhibitory effect on cell development. In contrast, KURE *et al.*¹³ have extracted from chick embryo a certain kind of nucleoproteins that has mitosis promoting effect. From these results it seems that among nucleoproteins there may exist some having antimitotic effect and others with promoting effect. Furthermore, the active site that shows antimitotic effect may originate in nucleosides and bases.

Quantitative relationship between mitosis and —SH groups can be estimated from the standpoint of phenomena,^{24,25,26} but there still remain many unsolved questions whether an increase in —SH group elicits mitosis or whether —SH quantity increases at the time when mitotic apparatus is formed.

It is questionable whether the cornin specifically inhibits only DNA synthesis and it has no effect on RNA synthesis. Whether the cornin has a tissue specificity as chalone—adrenalin complex reported by BULLOUGH *et al.*⁸ offers a great expectation for the development of future studies.

SUMMARY

Both the cornea and muscle cornins have no effect at all on oxygen uptake of tissue, and likewise they catalase affect activity not in any way. The cornea-cornin has an effect to reduce P/O ratio to about one half, but the muscle cornin does not show such an effect.

Both cornins decrease the incorporation of P^{32} into nucleic acid fraction and DNA synthesis. In the ultracentrifugal analysis of nucleic acids during development of sea urchin eggs, cornins inhibit the polymerization of nucleic acids.

In addition, both of these cornins depress the incorporation of P^{32} into DNA and ribosome RNA of regenerating rat liver.

Both cornins inhibit the increase of $-SH$ quantities before the cleavage of sea urchin eggs.

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