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Antimitotic action of cornin as a biologically active polypeptide. I. Biochemical properties of cornin*

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Abstract

We succeeded in the extraction of a substance from beef cornea and rabbit muscle, that markedly inhibits mitosis of sea urchin eggs. The substance extracted from beef cornea is non-dialysable and it can be separated into three fractions by DEAE-cellulose column. Although everyone of these fractions has an antimitotic action, that of fractions II and III is especially marked. These fractions are one of nucleoproteins that have adenine as base. The substance extracted from rabbit muscle is dialysable, and when it is fractionated through DEAE-cellulose column into three fractions, fraction I has no antimitotic effect but fractions II and III have it. Fraction II is one of nucleoproteins that have hypoxanthine as base. Carnin obtained from beef cornea or from rabbit muscle shows a typical protein wave, but after being treated with gas by passing oxygen through cornin solution the wave height is lowered. Carnin, however, is a very stable substance when kept dry in a desiccator.

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ANTIMITOTIC ACTION OF CORNIN AS A BIOLOGICALLY ACTIVE POLYPEPTIDE

I. BIOCHEMICAL PROPERTIES OF CORNIN*

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On severing the third cranial nerve of the cat, NISIDA *et al.*[22] noticed a curious phenomenon, a sudden contraction of dilated pupil in some hours after operation. It was demonstrated that this pupillo-contracting phenomenon was induced by a substance possessing a pupillo-contracting effect and that the substance was released from cornea. FUKUI[15] successfully isolated from the beef cornea this substance having pupillo-contracting effect and by its chemical analysis verified it to be a substance belonging to one of proteins, to which he gave a designation of "cornin". Later this cornin was found to have a stimulating effect on smooth muscle and a hypotensive action besides pupillo-contracting effect and a substance having similar properties was extracted also from rabbit muscle. This cornin was further verified to possess chemical properties different from those of biologically active polypeptides such as substance P, bradykinin, kalidin, and irin by the study of MIYAHARA[21] and KADO.[16] In addition, TOKUMOTO[27] found that the distribution of cornin *in vivo* resembled closely to that of substance P, whereas HINO,[14] NISIDA *et al.*[23] discovered that cornin has an antimitotic action on the early development of sea urchin eggs. In the course of study on biochemical properties and biological effects of cornin and substance P, it has been proved that cornin has a strong antimitotic activity while the substance P has a mitosis promoting effect.

We know that there are *in vivo* such tissues that are readily regenerated showing much mitotic index and also such that are non-proliferating showing hardly any mitotic picture, but each of these tissues, when cultured in an appropriate medium, will proliferate well. These facts have led us to an assumption that there might be substances *in vivo* that regulate cell division and cell proliferation.

This paper deals with biochemical properties of cornin, isolated from beef cornea and rabbit muscle, a substance that has been found to possess an anti-

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mitotic action by its bioassay.

MATERIALS AND METHODS

The material for the bioassay was sea urchin eggs that undergo synchronous division. The experiments were carried out with *Temnopleurus toreumatics* in summer (28°—32°C), and *Hemicentrotus pulcherrimus* in winter (6°—15°C), respectively. Matured eggs were collected by the electric stimulation or by an intracoelomic injection of 0.5 *M* KCl. The shed eggs were then immediately washed twice by decantataion and set aside as a stock suspension. Those eggs with the rate for fertilization of over 95 per cent were selected. Cornin was dissolved in sea water to which egg suspension was added as to make its final concentration 1×10^{-3}g/ml, 1×10^{-4}g/ml. ···. Unfertilized eggs were treated with the cornin solution for 10 or 20 minutes and then inseminated. The appearance of the first and the second cleavages against time was counted. As for the control, eggs were observed to undergo cleavage in sea water. For the experiments of removing fertilization membrane, silk cloth was used to remove the membrane 5—15 minutes after insemination, and these were placed in the test solution 20 minutes after insemination, and the first and the second cleavages against time were counted.

Cornin is extracted either from beef cornea or from rabbit muscle in boiling water for 10 minutes, cooled immediately, and alcoholic fractionation is done in a cold room at 0°C, which yields 70 per cent to 90 per cent cornin fraction. This is washed with absolute ethanol, acetone, methanol and ether and dried. The fractionation procedures are illustrated in Fig. 1. With each cornin extracted from the cornea and muscle, assays for protein, sugar, organic P, and nitrogen, ultracentrifugal analysis, paper electrophoresis and electrophoretic analysis with TISELIUS apparatus are done. In addition, by means of DEAE—cellulose column (by filling 5 g of DEAE—cellulose in the column of 1.6×50 cm) linear gradient elution of cornin is done in phosphate buffer and 0.1 *M* HCl, and with each fraction the

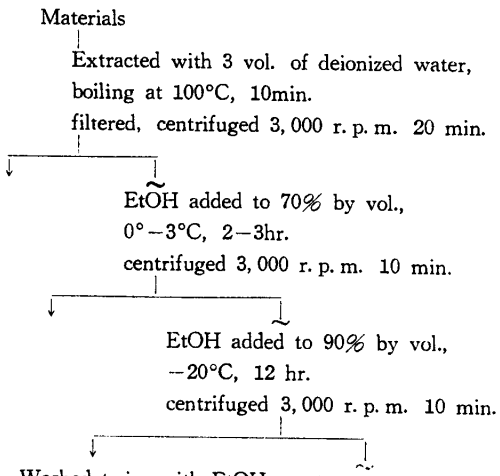

Fig. 1 Preparation Method of Cornin Extracted from Beef Cornea

ultraviolet absorption spectrum is determined, protein bond −SH group by polarographic analysis, and amino acid composition as well as basis of nucleic acids are estimated by paper chromatography and thin layer chromatography. Concurrently, biological effects of each cornin fraction are determined.

RESULTS

In Fig. 2 are shown the antimitotic effects of the cornea and muscle cornin on the first and the second cleavages. In this case, it is to be noted that the antimitotic effect is less when cornin is made to act after fertilization but its effect is stronger when it is made to act after the removal of fertilization membrane as shown in Fig. 3. This fact suggests that it is difficult for cornin to

Fig. 2

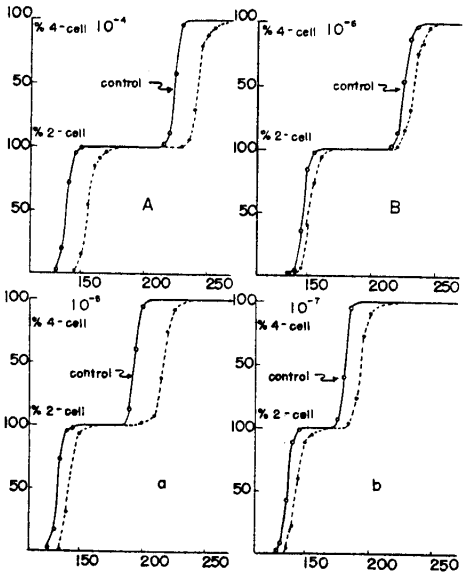

Fig. 3

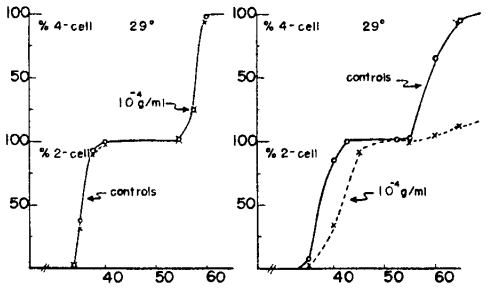

Fig. 2 Retarding Effect of Muscle (A. B) and Cornea-Cornin (a. b) on *Hemicentrotus* Eggs. Pretreatment for 20 Minutes. Ordinate shows percentage of 2-cell and 4-cell stage. Abscissa shows time after fertilization (in min.).

Fig. 3 Retarding Effect of Cornea-Cornin on Removing Fertilization Membrane of *Temnopleurus* eggs. Ordinate shows percentage of 2-cell and 4-cell stage. Abscissa shows time after fertilization (in min.).

permeate the fertilization membrane. When the cornin extracted from the cornea is dialysed against deionized water, the fraction that has an antimitotic activity is undialysable and the dialysable fraction shows only a slight mitosis promoting effect. In contrast, most of the fractions of the cornin from muscle are dialysable and these fractions have a strong antimitotic activity.

In the qualitative analyses of cornea and muscle cornin for protein, the cornea-cornin is positive to BIURET, xanthoprotein, SAKAGUCHI's and MOLISH's reactions, but the muscle cornin is negative as shown in Table 1. As for the qualitative analysis for sugar, the cornea-cornin is slightly positive to diphenylamine reaction while the muscle cornin is positive to phloroglucinol reaction as

Table 1 Qualitative Analyses of Cornea and Muscle Cornin for Protein

Tests	Cornea-Cornin	Muscle Cornin
Biuret reaction	+	−
Xanthoprotein reaction	+	−
Sakaguchi's reaction	+	−
Molish's reaction	+	−
Ninhydrin reaction	+	+
Liebermann's reaction	+	+
Neubauer-Rhode reaction	−	−
Bromphenol blue stain	+	+
T. C. A. precipitate	+	+
Protein-SH wave	+	+

Table 2 Qualitative Analyses of Cornea and Muscle Cornin for Sugar, and Organic Phosphate

Tests	Cornea-Cornin	Muscle Cornin
Benedict's test	−	−
Schiff's reaction	−	−
Feulgen's reaction	−	−
PAS reaction	−	−
Fehling's test	−	−
Seliwanoff's reaction	−	+
Phloroglucinol reaction	−	+
Diphenylamine reaction	±	−
Organic P	+	+

illustrated in Table 2. In further tests with acid or alkaline hydrolysate by paper chromatography and thin layer chromatography the cornea-cornin is adenine positive while the muscle cornin is hypoxanthine positive. The undialysable fraction of cornea-cornin shows one ultracentrifugal pattern, but by TISELIUS apparatus and paper electrophoresis it shows three patterns. The dialysable fraction of muscle cornin shows 5 patterns by the analysis with TISELIUS apparatus.

The results of the fractionation of cornea-cornin by DEAE—cellulose column are given in Fig. 4, those of ultraviolet absorption spectra in Fig. 5, and similar results of muscle cornin are shown in Figs. 6, and 7. The amino acid composition of each fraction is shown in Table 3. When polarograms of the cornea and muscle cornin are taken with hexamine-cobaltic chloride as the supporting electrolyte, a typical protein wave is revealed as illustrated in Fig. 8. However, when polarograms are taken after passing oxygen gas through each cornin solution for 5 minutes, there is a fall in the wave height.

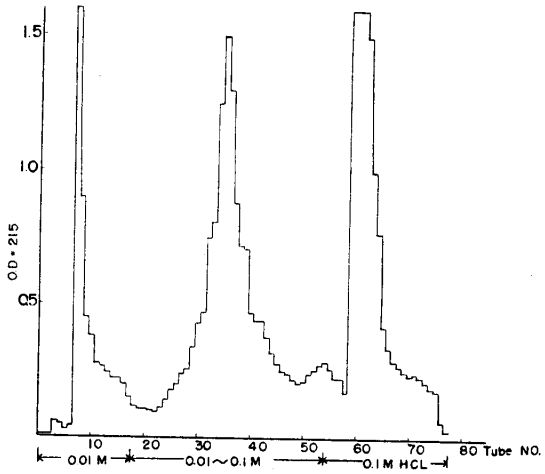

Fig. 4 Gradient Chromatography of Cornea-Cornin on DEAE-Cellulose Column

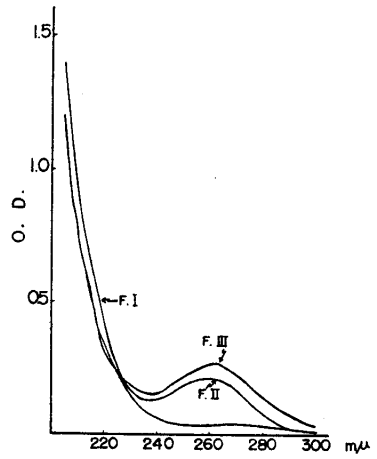

Fig. 5 Ultraviolet Absorption Spectra of Cornea-Cornin, Fractions I, II, III, Separated with DEAE-cellulose Column

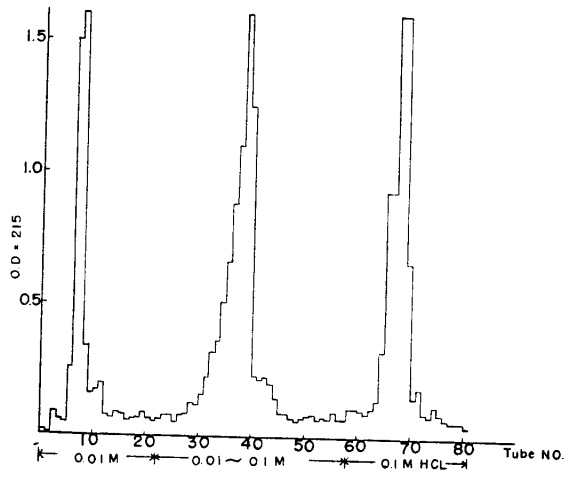

Fig. 6 Gradient Chromatography of Muscle Cornin on DEAE-Cellulose Column

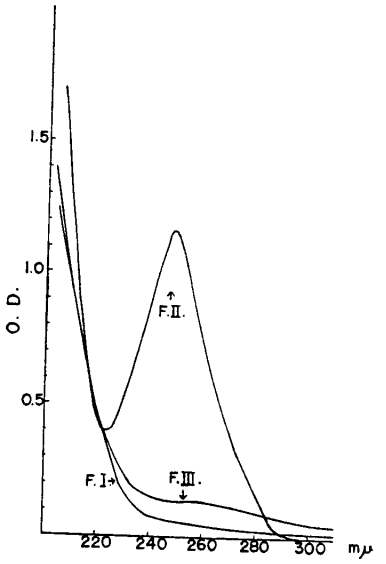

Fig. 7 Ultraviolet Absorption Spectra of Muscle Cornin, Fractions I, II, III, Separated with DEAE-Cellulose Column

Table 3 Amino Acid Composition of Each Fraction of Cornea and Muscle Cornin

Muscle-Cornin	
Fraction I.	Aspartic acid, Glutamic acid, Serine (or Glycine), Valine (or Methionine), Leucine (or Isoleucine), Alanine (or Threonine).
Fraction II.	Serine, Glycine.
Fraction III.	Aspartic acid, Glutamic acid, Serine (or Glycine), Valine (or Methionine), Leucine (or Isoleucine), Alanine (or Threonine).
Cornea-Cornin	
Fraction I.	Aspartic acid, Glutamic acid, Serine, Glycine, Threonine, Alanine, Leucine (or Isoleucine), Valine, Proline, Cystine, Lysine (or Arginine).
Fraction II.	Aspartic acid, Glutamic acid, Serine, Glycine, Threonine, Alanine, Leucine (or Isoleucine), Phenylalanine, Valine, Tyrosine, Proline, Cystine, Lysine, Histidine, Arginine.
Fraction III.	Aspartic acid, Glutamic acid, Serine, Glycine, Threonine, Alanine, Leucine (or Isoleucine), Phenylalanine, Valine, Proline, Cystine, Tyrosin, Lysine, Histidine, Arginine.

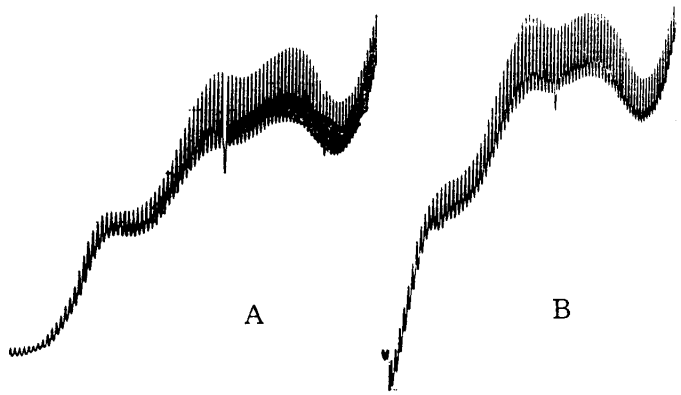

Fig. 8 Polarograms of Cornea-Cornin (A) 6×10^{-5}g/ml, and Muscle Cornin (B)6×10^{-4}g/ml

DISCUSSION

With a remarkable advance in the knowledge of biochemistry about nucleic acids and under the pressing need for control of cancers as well as cancer cell proliferation, the study on cell division has come to be one of the most important objects in the field of natural sciences.

In a series of works SZENT-GYÖRGYI *et al.*[9,10,26] have succeeded in isolating from thymus, aorta and tendon a substance, designated as "promine" that acts as to promote the cell division and another substance, "retine", that acts inhibi-

torily on the cell division. From what they have reported, it may reasonably be deduced that there exist two substances in living tissues, one that promotes the cell division while the other inhibits the division, and that the balance of these two substances *in vivo* would regulate the mitotic mechanism.

Such an idea has been entertained for a considerably long period of time, and therefore, by selecting specifically those antimitotic substances possessing similar biochemical properties have been extracted from living tissues, we shall attempt to compare them with this antimitotic substance, "cornin."

HEILBRUNN *et al.*[11,12,13] has extracted a substance from ovaries of starfish, fish, cow, pig and lamb, that inhibits cell division. They state that, since this substance is dialysable and protein-reaction negative and sugar-reaction positive and from its ultraviolet absorbancy, it is a hepalin-like substance. It differs from each fraction extracted from cornea and muscle in that it is not protein-reaction positive as is "cornin." MENKIN[20] has also extracted from *Arbasia* eggs an antimitotic substance that is heat stable, indiffusible, BIURET and ninhydrin reactions positive, and has absorption band at 265 mμ to around 270 mμ and he considers it to be a nucleoprotein. It resembles rather closely the fractions II and III of the cornea-cornin but differs from them in its absorption peak. Likewise its absorption peak is different from the fractions of muscle cornin. WOLFSON[28] has extracted a retarding factor from sea urchin eggs, ovaries, testes and gut, but as its chemical properties are unknown, it is impossible of comparison with cornin. "Retine", the fraction isolated by SZENT-GYÖRGYI *et al.* is likewise not a subject of comparison because its biochemical properties are unknown, but it can be said that "retine" is a very unstable substance while "cornin," though its aqeuous solution is unstable, in a dried form, its activity is preserved for over two months. Histones as reported by MIRSKY *et al.*[1] have the properties very similar to those of the fraction III of cornea-cornin. In our experiments with arginine-rich and slightly lysine-rich histones, however, we find that histone agglutinates cells while cornin has no effect on sperms, and in addition, histone differs from cornin in that it not only agglutinates spermatozoa but also its minimum antimitotic effective dose is 10^{-6}g/ml. RN-ase is heat stable and has antimitotic effect, but it differs from cornin in its absorprion peak at 280 mμ. Further, STICH[24] and GOUTIER *et al.*[7,8] reported about a microsome fraction from normal rat liver, that possesses antimitotic effect, but its biochemical properties are unknown. The aqueous extract of epidermis, as reported by BULLOUGH *et al.*[5] likewise shows antimitotic effect only in the form of adrenalin-complex. LALLIER[17] reported that some of bases, nucleosides, and nucletides themselves had antimitotic activity. It may be said that the active site of cornin might be located in these substances, but there is no need of comparison between cornin and these substances.

As has been pointed out in the foregoing, the antimitotic substance is distributed in various tissues in a variety of forms and it is not a single substance. As mentioned in a recent integrated review by LATTRE[18], BRACHET[4], SWANN[25], BARTH[2], BASS[3], LEVI-MONTALINI *et al.*[19] and DUSTIN[6], antimitotic substances are of many kinds, and roughly they can be divided into two large classes, one that inhibits the mitotic apparatus formation and the other that inhibits nucleic acid synthesis. As for cornin it is difficult to say to which one of the two classes it should be allocated, but morphologically it seems to inhibit the formation of mitotic apparatus and it has been verified biochemically that cornin decreases the P/O ratio, and inhibits the uptake of P^{32} into nucleic acid and DNA synthesis.

SUMMARY

We succeeded in the extraction of a substance from beef cornea and rabbit muscle, that markedly inhibits mitosis of sea urchin eggs. The substance extracted from beef cornea is non-dialysable and it can be separated into three fractions by DEAE–cellulose column. Although everyone of these fractions has an antimitotic action, that of fractions II and III is especially marked. These fractions are one of nucleoproteins that have adenine as base. The substance extracted from rabbit muscle is dialysable, and when it is fractionated through DEAE–cellulose column into three fractions, fraction I has no antimitotic effect but fractions II and III have it. Fraction II is one of nucleoproteins that have hypoxanthine as base. Cornin obtained from beef cornea or from rabbit muscle shows a typical protein wave, but after being treated with gas by passing oxygen through cornin solution the wave height is lowered. Cornin, however, is a very stable substance when kept dry in a desiccator.

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