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REGULATORY ACTION OF DEOXYRIBONUCLEIC
ACID FRACTIONS ON CLEAVAGE OF
ARBACIA EGGS

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DNA AND CLEAVAGE

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A B S T R A C T

This study deals with the effect of DNA derivatives on cleavage of sea urchin eggs obtained during the summer from the ovaries of Arbacia punctulata which occur on the Lebanese coast. The DNA used was highly polymerized calf thymus DNA. It was treated with the enzyme DNA-ase to break it into smaller fragments. These were then fractionated on an "Ecteola"- cellulose column and several fractions were collected by eluting the column with a series of NaCl solutions of various concentrations. After correcting the salinities of the collected fractions and performing some chemical and physical tests on them, they were tested for their effect on the cleavage of Arbacia punctulata eggs.

The results obtained suggest that two components occurred after digesting the DNA with DNA-ase. One component was shown by a series of experiments to be inhibitory to cleavage, while the other component was shown by another series of experiments to be stimulative to cleavage. Further studies are required to reveal the chemical nature and the mechanism of action of these two components.

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I N T R O D U C T I O N

The phenomenon of cell division is one of the most interesting events in the life history of the cell. The process is many sided and its study has attracted attention from various fields of science including cytology, physiology, biochemistry, biophysics, genetics, and medicine. Much work has been done in the hope of understanding the mechanism of the process, to know what sets it on, how it proceeds, the conditions and changes accompanying every step of it, and phenomena of misdivision and abnormal division especially important in the study of cancer.

The literature on the subject is voluminous but much of it is speculative. Only the most basic facts and the important experimental aspects of the study will be presented here.

The earliest among the methods of study of the subject has come from cytology aiming at finding the morphological changes in the cell during mitosis.

Beside the morphological studies mitosis also has been studied by biophysical methods mainly in relation to changes in viscosity and surface rigidity of the cell. Heilbrunn (1952;1956) emphasizes the fact that protoplasm is a colloid the properties and activities of which are tied to its colloidal nature. Using carcinogenetic substances on the one hand and chelating substances on the other hand he

found that the former induced a temporary gelation raising the viscosity of the protoplasm, while the latter substances prevented gelation and dropped the surface rigidity of the cell resulting in inhibition of mitosis. Other treatments such as X-ray irradiation, cold, heat, etc., which inhibit mitosis also bring about a drop in viscosity of the cell. Parthenogenetic treatments primarily raised the viscosity of the cell in advance to division. These observations led Heilbrunn (1952) to the formulation of the "theory of stimulation and response" which assumes that stimulation of the cell releases Ca^{++} from the cell's cortex bringing about gelation of the internal cytoplasm. This gelation is necessary for the formation of the mitotic spindle. When the latter is formed the viscosity of the cell drops again. Support for the idea of the formation of the mitotic spindle together with gelation comes from Rapkine (1931) and Mazia (1956). The first observed that gelation is accompanied by an increase in the oxidative processes due to oxidation of -SH groups in sulfahydryl containing compounds (mainly proteins in this case) to S-S, and the second concluded from the chemical nature of the mitotic apparatus (a protein rich in S) that it is formed by oxidation of SH groups in a soluble protein to S-S forming an insoluble protein.

The above process of gelation must be preceded by a process of nuclear breakdown. Goldstein (1953) gave this process an interpretation on the basis of the "theory of stimulation and response". He concluded that stimulation

releases Ca^{++} from the cortex of the cell. The Ca^{++} stimulates a proteolytic enzyme system in the internal cytoplasm which acts on the nuclear membrane to break it while at the same time liquifaction takes place in the cytoplasm. Heilbrunn (1956) gives evidence that it is possible to have a clotting or a liquifaction due to the action of Ca^{++} .

Finally Heilbrunn (1956) explains the formation of the cleavage furrow as due to the weakening of the spindle at the equatorial plane. Breaking of the spindle which is usually in tension, pulling the astral rays which in turn pull the cortex of the cell, changes the equilibrium of forces, and requires the establishment of a new cleavage of the cytoplasm through the equatorial plane.

The Energetics of Mitosis

The process of mitosis requires expenditure of a certain amount of energy which is obtained from processes such as respiration. Scholander et al, (1952) working on respiration of single cells during cleavage, found that in some kinds of cells there was no increase in the rate of respiration during mitosis, while in other kinds there was a cycling of respiration with respiration increasing at the phase of cytoplasmic cleavage. In any case, energy for mitosis can be obtained either from glycolysis (anaerobically) as in the frog's egg, tumors, some embryonic tissues, and some adult tissues (Riesle 1958), or from the Krebs' Cycle (aerobically) as in the sea urchin egg and many tissues (Krahl 1950).

What are the raw materials and what are the products of the above mentioned energy yielding processes which give energy to mitosis? This question has been studied by several investigators. Bullough (1952), working on mitosis in epidermal tissue of the rat, found that the process requires glucose as an energy source, energy being obtained through oxidation of glucose in glycolysis and the Krebs's Cycle with yield of high energy phosphorus compounds which seem to play an important role in mitosis. This has been revealed (Barnett 1953) by dissociation of respiration from phosphorylation by treatment with such poisons as DNP (dinitrophenol) and other nitro and halo-substituted phenols. Inhibition of mitosis takes place due to inhibition of phosphorylation although the rate of oxygen consumption in some cases increases three or four times the normal. This inhibition has been found to be reversible by addition of ATP. Runnstrom and Kriszat (1950) found that ATP improves fertilization and segmentation of sea urchin eggs. ATP also has been conceived as necessary for the process of separating the chromosomes in anaphase by effecting contraction of the protein of the spindle in the mitotic apparatus in a way similar to its action on actin, the protein of muscle (Mazia 1957).

Beside the importance of the Krebs's Cycle for providing enough high energy phosphorus compounds for cleavage, it has been shown in the case of sea urchin eggs to have another independent action in regard to cleavage as shown by the in-

ability of ATP to reverse inhibition due to azide or malonate which do not interfere with phosphorylation. This inhibition has been reversed by Kreb's Cycle intermediates (Barnett 1953) indicating that division in this case is intimately related to a functioning Kreb's Cycle, the function of which cannot be completely accounted for on the basis of the production of high energy phosphorus compounds (Barnett 1953).

The Inhibition and Initiation of Cleavage

Much current research is being done on the problem of inhibition and initiation of cell division. The aim of such studies is to find biochemical substances which produce inhibition or initiation of cell division. Antimitotic factors which resemble heparin have been isolated mainly from ovaries of invertebrates and vertebrates (Heilbrunn, Wilson, and Harding, 1951; Heilbrunn et al 1954; Heilbrunn and Wilson, 1956). Allee et al (1942) have isolated a retarding factor from the ovaries of sea urchins which seemed to be a nucleoprotein. Later work by Menkin (1956) on ovaries of sea urchins revealed the presence of a non-dialyzable fraction in the ovaries, which had a retarding effect on division of sea urchin eggs. Further studies by Menkin et al (1959) indicated that the retarding factor was equally effective on division of fertilized eggs of the clam, Spisula solidissima. The active material was found by Menkin (1959) to be a polynucleotide.

Other inhibitory or retarding substances include ribonucleotides as found by Berrian and Dornfeld (1950) who worked on the germinal epithelium of the rat. Hughes(1952) tested purines and related substances on cells in chick tissue cultures. He found adenine, 2-6 diaminopurine, xanthine and cytosine to have varying strengths for inhibiting mitosis at metaphase or a little beyond metaphase, causing reconstitution of the nuclei before telophase with an effect on the spindle. Hughes classified the above compounds as metaphase inhibitors. He also classified nucleosides and nucleotides of which he tested adenosine, adenylic acid and ATP, as preprophase inhibitors of mitosis.

Deotto (1954) also has found that DNA and RNA injected into regenerating liver inhibited cell division. Moreover DNA extracted from one species of sea urchins was found by Mazia (1949) to inhibit division of eggs of the same species. Butros (1959) noted that some DNA preparations, when degraded, give inhibitory effects on cleavage if inorganic phosphate was split. Undegraded highly polymerized DNA was also inhibitory to cleavage, presumably due to interference with "surface activities" of the eggs.

On the other hand substances which have been found to initiate, stimulate, or accelerate cleavage have also been described. In the same extract of sea urchin ovaries in which Menkin found the cleavage retarding factor, he also found a cleavage accelerating factor (Menkin 1956). This factor like

the retarding factor was also effective on cleavage of ova of sea urchins and ova of the clam Spisula solidissima (Menkin et al 1959). Characterization of the accelerator factor suggested that it was a dinucleotide which had uridylic acid as one component (Menkin 1959). In the same study DNA, ATP, uracil and cytosine were tested on ova to determine their effects on cleavage. Uracil was found to be accelerating to cleavage, cytosine and DNA showed no effect, while ATP had no consistent effect, sometimes being accelerating while at other times it was retarding or had no effect.

Allee et al (1942) also reported the existence of an accelerating cleavage factor which had characteristics of a nucleotide.

Another type of cleavage initiating compound was found by Shaver (1953) who worked on initiation of cleavage in the frog's eggs. This compound was found to be rich in sulfahydryl groups.

A potent stimulant of cell division was more recently reported by Miller et al (1955). It was called Kinetin and was identified as 6-aminofurfuryl purine.

Finally the most recent work has been reported by Butros (1959) who tested under different conditions the effect of three DNA preparations from various sources on the cleavage of sea urchin eggs obtained from the ovaries of sea urchins during summer. At this season the ovaries are less than half as large as during the Winter and Spring. Such

eggs were found to have a very low percentage of cleavage in natural sea water but were stimulated by degraded DNA except that which seemed to give inorganic phosphorus. Deoxypolynucleotides which are the main products of DNA degradation by DNA-ase (and supposedly by autoclaving) were regarded by Butros to be responsible for the stimulating action on cleavage.

One other finding in relation to DNA was made by Horstadius et al (1954) who noted that DNA extracted from sea urchin sperms and from calf thymus gland activated unfertilized sea urchin eggs if it entered the eggs and mixed with the cytoplasm. The eggs started cleavage but finally cytolized. When calfthymus DNA was injected but did not mix with the cytoplasm of the eggs, several asters formed in the egg cytoplasm but no cleavage occurred.

The above findings of inhibitory and initiating substances have given substantial support to the idea that in the cell there is a mechanism for coordinating a balance between inhibitory and stimulating substances inside the cell. Among these studies the findings of Butros (1959) seem to be of special importance in view of the biological importance of DNA as probably representing the primary hereditary material. Any understanding of the relation between nucleic acids (particularly DNA and its derivatives) to cleavage has not yet been arrived at. However, several things strongly suggest that these compounds play an important part in the process of cell division. DNA concentration doubles in the

cell during mitotic activity as found by Pasteels and Lison (1950). In abnormal conditions such as in tumors the DNA concentration often increases in correlation with increase in the number of chromosomes in the cells (Mellors, 1956). Fautrez et al (1955) showed that during regeneration of kidney tubules increase in mitotic activity was correlated with increase of DNA content in the interphasic nuclei. Brachet (1950) observed that DNA and RNA concentrations increase in the young blastema. According to Marshak and Walker (1945) DNA turnover increases in actively proliferating liver and injected chromatin stimulates mitosis.

Purpose of the Present Study

This study is concerned with the effect of deoxypolynucleotides on cleavage of sea urchin eggs. It is an extension of the work started by Butros (1959) the results of which suggested that among the products of digestion of DNA by DNA-ase there was a fraction which stimulated cleavage of naturally inhibited sea urchin eggs. The study aimed at confirming the work of Butros and locating the suspected DNA digestion product which stimulates cleavage if such a special component exists.

MATERIALS AND METHODS

The methods used in this study are primarily the same as those used by Butros (1959). The deoxypolynucleotides were obtained in fractions after chromatography of a DNA solution digested with DNA-ase on an "Ecteola"-cellulose column prepared according to Peterson and Sober (1956). This column has been found by Bendich (1956) to have a high resolving power for DNA and to maintain the integrity of the fractionated DNA with consistency in results. The fractions obtained were tried as to their effect on the cleavage of eggs of the sea urchin Arbacia punctulata during summer.

Digestion of DNA by DNA-ase

Highly polymerised calf thymus DNA was digested with DNA-ase.¹ The procedure of digestion was a modification of the procedure of Sinsheimer (1951): 50 mgs. DNA were dissolved in 30 mgs. of distilled water. To this 0.09 gms. $MgCl_2$ and 0.072 gms. of sodium acetate (NaAc) were added followed by the addition of 2 mgs. of DNA-ase. The pH was adjusted to 7 by titration with NaOH(0.01 N). The $MgCl_2$ and NaAc added provided Mg^{++} as an activator for the enzyme and an acetic acid - NaAc buffer for controlling the pH. The digestion mixture was then incubated in water at 37°C with constant shaking. The digestion period lasted 10 hrs. during

1. Both the DNA and the DNA-ase were prepared by the Nutritional Biochemical Corporation.

which the pH of the digestion mixture was measured at intervals and corrected to 7 by titration with more 0.01 N NaOH whenever the pH deviated from 7. After this period several tests for the occurrence of digestion were done on the digest. The tests, as described by Schmidt (1955) are the following:

1. Turbidity test. 1 volume of the digest with 2 volumes of glacial acetic acid. Absence of turbidity indicated digestion of DNA.
2. Increase in U. V. absorption. An increase in U. V. absorption by 40% compared to undigested DNA occurs when digestion occurs.
3. Drop in the viscosity of the DNA solution when digested. This was measured by an Ostwald Viscosimeter by measuring the time it takes a certain volume of the tested solution to pass through a given distance in the viscosimeter and comparing this time to that obtained for an equal volume of DNA solution.

The digested DNA solution was then ready for fractionation on the Ecteola cellulose column.

Preparation of the Column

The column used was the Ecteola anion exchanger column. The powder of the column was prepared for chromatography from Whatman standard grade ashless cellulose powder.² The method of preparation was according to the procedure of

2. Prepared by W. and R. Balston Ltd., England.

Peterson and Sober (1956) while adaptation of the column to the study of DNA follows the procedure of Benedich (1956). The following is the procedure of preparation:

A solution of 60 gms. of NaOH in 150 ml. of water was added to 60 gms. of the cellulose powder with stirring to get a well blended mixture. This was then immersed in an ice-bath for 30 min. Then a mixture of 140 cc. of triethanolamine (the amount of triethanolamine being increased four-fold)³ and 60 ml. of epichlorohydrin were added and mixed well with the powder suspension. The mixture was then immersed in a 75 - 80° oil bath for 30 mins. and stirred at intervals.

After heating, the mixture was cooled in an ice bath and "250 ml. of 2 M NaCl were added in portions and mixed well with the powder after each addition to obtain a smooth suspension."⁴ The product was then filtered " with gentle suction in a sintered funnel, and the filter cake was washed with 1 N NaCl until the emerging filtrate was no longer deeply colored. The product was then resuspended on the filter in sufficient 1 N HCl (about 350 ml.) to make a strongly acid suspension and this was immediately filtered. Successive washing with 250 ml. portions of 1 N NaOH, 1 N HCl, and 1 N NaOH followed. The cake was tamped down and pulled dry to

3. Fourfold of the amount used by Peterson and Sober. This according to Benedich (1956) increases the capacity of the column for DNA.

4. Peterson and Sober, 1956, J. Am. Chem. Society vol. 78 p.754.

the cracking point between washings but was not rinsed with water. Finally the cake was again suspended in 250 ml. of 1 N NaOH, then transferred to a large vessel and diluted with water to 3 liters. After standing overnight the cloudy supernatant liquid was decanted, and the sediment was washed 5 or 6 times by decantation to remove particles that would not settle. The final supernatant liquid was almost clear and was free of alkali. The sediment was filtered to remove as much water as possible, then washed with about 50 ml. of ethanol in three portions, the last one absolute ethanol. As much of the ethanol as possible was drawn off on the filter then the partially dried product⁵ was completely dried by evaporating the alcohol in vacuo.

When the powder was ready 10 gms. of it were then suspended in a 0.01 M phosphate buffer and the suspension was added to a glass column. The column was then washed successively with equal volumes of 0.5 N NaOH, 0.5 HCl, and 0.5 N NaOH and finally washed well with distilled water.

Chromatography on the Column

The column was then saturated with 0.1 M NaCl and the solution of the digest was added to it. The solvent passing through the column was collected and tested for DNA by diphenylamine and was always found to be negative. Elution of the adsorbed DNA digestion products was then carried

5. Peterson and Sober, 1956, J. Am. Chem. Society vol. 78 p. 753

out by adding successively 25 ml. of the following ten solutions of NaCl; 0.1 M, 0.3, 0.5, 0.7, 0.9, 1.25, 1.5, 1.75, 2 M at pH 10.5.

The column was then washed with distilled water and saturated with 0.1 M NaCl and the fractions which were collected were examined spectrophotometrically or with diphenylamine to determine their relative content of the nucleic acid derivatives obtained on digestion.

Paper Chromatography of the Fraction

Paper chromatography was one method to characterize the contents of the fractions and to know how many components of different chromatographic behaviors were present. According to Sinsheimer (1951,1955) the products of digestion of DNA by DNA-ase were mainly deoxypolynucleotides. A method for separating deoxypolynucleotides on paper chromatograms had been worked by Chargaff (1951) based on a method for separation of ribonucleotides applied by Magasanik et al (1950). The method used in this study was basically the same as used by Chargaff except for the use of Whatman No.1 filter paper instead of Shleisher and Shull paper which was un - available.

Procedure: Isobutyric acid and 0.5N NH_3 were used as the solvent system. They were mixed in the proportion of 10;6 respectively. The pH was 3.6. A small beaker containing the mixture was put in the bottom of the chromatography jar some-

time before starting the separation so as to saturate the atmosphere of the jar with the solvent. Equally measured amounts (usually 0.025 ml) of each fraction collected from the column were spotted on Whatman No.1 filter paper and irrigation by descending chromatography was carried out for 12 - 15 hrs. The paper was then taken out from the jar and the place where the solvent had reached on it (solvent front) was marked. The paper was then left to dry at room temperature. The spots were then detected on the paper either by use of a U.V. mineral-light lamp in a dark room or by carrying out the Dische reaction on the paper as described by Buchanan (1951). In the first method the spots of DNA derivatives appeared as dark areas on the paper due to their absorption of U.V. light. The positions of those dark areas on the paper were marked with pencil. In the second method the paper was sprayed with a solution of diphenylamine and was then put in an oven at 90° C for 10 - 15 minutes. While in the oven the paper was covered by two plates of glass so that it would not be burned at that high temperature. The DNA derivatives on the paper reacted with diphenylamine giving blue spots wherever appreciable amounts were present. The R_f value for each spot was then calculated by dividing the distance which the spots travelled on the paper by the distance moved by the solvent. The results are tabulated in Table I.

Preparing the fractions for testing on the eggs.

After collecting the fractions from the column as described earlier it was necessary to adjust the salt and mineral composition of each fraction to make it equal to that of sea water. The following two procedures were applied to accomplish this.

Procedure 1. This was used with experiments 1-14. A formula for the composition of sea water given by Zobell (1946) was used as a standard and the mineral and salt compositions of the fractions and the controls were adjusted according to it. The salinities of the fractions were measured by titrating 2cc of each fraction with 0.1 N AgNO_3 . The amount of NaCl per c.c. of each fraction was calculated after titration. Fractions found to be at a lower salinity than sea water were adjusted by adding NaCl, while those at a higher salinity were diluted. In both cases the amount of NaCl was adjusted making the amount per 9cc of each fraction equal to the amount per 10cc of sea water according to Zobell's formula. To 9cc of each fraction in a 50 cc beaker was added 1cc of a ten times concentrated sea water lacking NaCl and prepared according to Zobell (1946). The salinity and mineral composition of the fractions were then equal to that of sea water. The control was then prepared from 9cc distilled water + 1cc of the ten times concentrated sea water lacking NaCl + 0.2348 gms of NaCl.

Procedure 2. This was applied with experiments 15-22 accord-

ing to a suggestion by Dr. Tyler (1959). About 20cc of each fraction were put in cellophane bags and the bags were tightly closed and dipped into beakers containing natural sea water. Dialysis was carried on for two days with several changes in the sea water. Natural sea water was used here as a control.

Dialysis of the fractions

Experiments 14 and 17 were side experiments which were performed to test the effect of the derivatives of dialysis on cleavage of Arbacia eggs. In experiment 14 dialysis was carried against distilled water for two days and the solutions outside the bags and those inside the bags from fractions 2,3 and 4 were tested on the eggs after correcting their salinities and mineral compositions by procedure 1 described in the preceding section. In experiment 17 fractions 2,3,4 and 5 were dialyzed against natural sea water and only the solutions outside the bags were tested on eggs. The results of both experiments 14 and 17 are reported in Table IV.

Testing the fractions on the eggs.

After the salt concentration of the fractions was adjusted to that of sea water the pH of the fractions was corrected where necessary to 8.2 which was the pH measured for natural sea water. When the pH was not 8.2 it was always below it. The pH was raised to 8.2 by adding some drops of a solution of 0.1% NaHCO_3 . The measurements of pH were done with a Lamotte pH meter in which one uses color indicators

and compares against the colors of standards.

Having corrected the pH the solutions were finally ready to be tested on the eggs. The beakers containing the experimental and control solutions were put in a water bath at room temperature away from direct sunlight to avoid variations in temperature. Sperms and eggs were then collected from *Arbacia* following basically the methods described by Just (1939). The procedure was as follows:

Sea urchins were taken from an aquarium and with clean scissors a cut was made around the peristome to remove Aristotle's lantern. The perivisceral fluid which is supposedly inhibitory to cleavage was decanted and the sea urchin was put on a syracuse dish in order to shed its eggs or sperms. The shed eggs and sperms were observed under the microscope. The best eggs were chosen for use on the basis of normality of shape and absence of a high number of immature eggs as judged by the presence of a germinal vesicle in the immature eggs. The chosen eggs were washed with sea water, then concentrated in the syracuse dish in which they were shed. With clean pipette eggs were taken from the dish and transferred in equal amounts (5 drops) to the beakers containing the experimental and control solutions. The eggs were incubated in the solutions for 1 hour before fertilization. They were then fertilized with a freshly prepared sperm suspension. Samples of the fertilized eggs were observed under the microscope to insure that the eggs were fertilizable, judged by presence of an elevated fertilization membrane. Division of the fertilized

eggs usually started after 50 minutes and about 10 minutes later they were transferred to vials containing formalin for fixation. The fixed eggs from the experimental and control solutions were counted under the microscope to determine the proportion of cleaved to uncleaved eggs. The total number of eggs counted was at least 100. The results are reported as % of divided eggs in Table III. The significance of the results was determined by using the following formula discussed by Dahlberg (1948).

$$E(D) = \sqrt{\frac{P(100-P)}{\text{total}} + \frac{Q(100-Q)}{\text{total}}}$$

E(D) represents the standard error of the difference, P and Q the % of divided eggs in the experimentals and control respectively. When the difference between Q and P exceeds three times the value of E(D) the results are reasonably significant.

R E S U L T S

1. Paper Partition Chromatography

Paper chromatography was used as an accessory method in the hope that it might throw some light when conclusions on the effect of DNA digestion products are made. The results can only indicate the number of constituents of different chromatographic behavior in each fraction and consequently would indicate to some extent differences in the composition of each fraction from the other fractions. The results obtained for the few experiments that were done are reported in Table I. They show variations in the number of constituents of different chromatographic behaviors in the fractions of each digestion made. This indicates that there were qualitative differences between the constituents of the different fractions.

T A B L E I.

Measurements for the R_f values of spots of DNA derivatives detected on paper chromatograms by reacting with diphenylamine.

Digestion No.	Fraction No.	No. of Spot.	R_f value of Spot.
1	1	-	-
	2	1	0.22
	3	{ 2	0.20
		{ 3	0.274
	4	4	0.17
5	-	-	
2	1	-	-
	2	1	0.13
	3	{ 2	0.13
		{ 3	0.261
		{ 4	0.32
4	5	0.184	
5	-	-	
3 1st Trial	1	-	-
	2	-	-
	3	{ 1	0.047
		{ 2	0.11
		{ 3	0.173
4	4	0.119	
5	-	-	
3 2nd Trial	1	-	-
	2	-	-
	3	{ 1	0.157
		{ 2	0.23
	4	3	0.14
5	-	-	

2. Measurements of DNA concentration in the fractions

These measurements are reported in Table II A and B. They were taken only for fractions from some of the digestions made. The diphenylamine test was used in most cases while spectrophotometric measurements were used occasionally. The results can be used to indicate any quantitative differences in concentrations of DNA derivatives in fractions of the same digestion and in corresponding fractions of different digestions. Such results give some idea of the reproducibility of the column and throw some light on how would variation in the concentrations of DNA derivatives in the fractions influence the percentage of cleavage.

Table II (A)

Concentration of DNA derivatives as indicated by intensity of color of Diphenylamine tests.

Fraction	D ₁ ⁶	D ₃	D ₄	D ₅	D ₆	D ₁₁	D ₁₂
1	-	-	-	+	+	-	+
2	++	+	++	+++	++	+++++	+++++
3	+++	++++	++++	+++	+++	++++	++++
4	+	++	+++	++	++	+++	+++
5	-	-	+	+	+	++	++
6	-	-	-	-	?	+	+
7	-	-	-	+	?		
8	-	-	-	-	-		
9	-	-	-	-	-		
10	-	-	-	-	-		

Table II (B)

U.V. absorption of DNA derivatives at 260 mu.

Fraction	D ₁	D ₂	D ₃
1	0.003	0.173	0.013
2	0.495	0.368	1.225
3	0.918	1.22	1.52
4	0.249	0.266	0.209
5	0.0057	0.024	0.031
6	little below 0	0.018	below 0
7	-5	0.052	0.084
8	0.002	0.029	below 0
9	-14	0.190	
10	-18	-2.5	

6. D. stands for the digested sample. The number shows the order of the sample among the twelve digestions made.

3-The Effect of Fractionated DNA Digestion Products On Cleavage

As shown in Tables III and IV twelve digestions of DNA were prepared by breaking DNA with DNA-ase as described earlier. Digestion 4 was not tried on cleavage and so no results were reported for it. It could be seen from the Tables that the material used in certain experiments was derived from the same preparation as that used in other experiments, (e.g. the same material was used in experiments 4, 5 and 6; 7, 8 and 9; 10 and 11, etc). The only differences between such groups of experiments lie then in the time the material was applied to the eggs (see dates) and also differences between the animals used.⁷

In the footnote for Table III the degree of dilution of several fractions in several experiments is given. Dilution was resorted to after observing the occurrence of cytolysis in the eggs in some fractions of experiments 1-4 and 7 (Table III). The cytolysis was most pronounced in fractions 2 and 3. After dilution no cytolysing effects appeared and inhibition of cleavage persisted in several fractions in all the experiments done using artificial sea water as a control i.e. experiments 1-13 (Table III). It is noteworthy that although the material tested on the eggs was diluted in some experiments, the inhibitory effect on cleavage was still persistent and in some experiments it became even stronger. In experiment 5 for example although

7. Variation in the reproducibility of the column may be an additional factor responsible for differences in results of various experiments.

T A B L E III

Percentages of Cleaving Arbacia Eggs in Sea Water
Containing DNA Derivatives.

Experiments 1-13 using artificial sea water.⁸
Salinity adjusted by titration and addition
of minerals in amounts required.

			% Cleavage							C o n t r o l
Digestion NO.	Experiment	D A T E	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	
1	1	July 21	0*	0*	44	41	33	27.3	47	36
2	2	" 24	90	0*	0*	90	90	90	90	95
3	3	" 30	100	0*	0*	0*	100	100	100	97
5	4	Aug. 10	5.3*	0*	2.2*	1.6*	6*	5*	5*	50
"	5	" 13	2.3*	0*	1.2*	1.5*	2*	2*	2*	75
"	6	" 15	30	30	30	30	30	30	30	50
6	7	" 10	-	5.6*	15.6*	4.2*	86*	49*	84	72
"	8	" 13	1*	0*	1*	3*	6*	11*	11*	55
"	9	" 15	30*	30*	30*	30*	30*	40	40	50
7	10	" 22	6.3*	16.3*	18*	29*	39	40.6	47.4	59
"	11	" 28	78.7*	0*	11.11*	27.6*	15.8*	45*	33*	100
8	12	Sept. 1	14.2*	0*	7.1*	21*	20*	35.1*	53.6*	100
"	13	" 1	14*	3*	9.5*	14.5*	17.5*	20*	11.4*	85
Aver- age			31.5*	6.5*	13*	22.6*	36.6*	38*	42.6*	71

Experiments 15-22 Using dialysis against natural
sea water for adjusting the salinity of the fractions

9	15	"	7	51	95*	95*	45	40	-	40
"	16	"	7	44.2	67.6*	60*	50	43.4	-	30
10	18	"	8	29.9	47	53	54.5*	54*	-	37.9
"	19	"	8	100*	100*	100*	100*	98*	98*	30
11	20	"	14	35.2	52.3	77*	81*	69.9*	70*	43
12	21	"	15	97*	94*	98*	95*	30*	30*	2
"	22	"	16	100*	98*	100*	55	54	54	50
Aver- age				51.7*	79.4*	83*	55.1*	43.7*	63*	23.2

* Asterisks indicate cases where there were significant differences between the percentages of cleavage of experimentals and controls.

8. Compared to the concentrations of the original preparations fractions 1-4 in experiments 5,6,8, and 9 were diluted 5 times. Fraction 5 was diluted twice and fractions 6 and 7 were undiluted. In experiments 10-13 all fractions were diluted twice.

fractions 1, 2, and 3 were diluted 5 times in comparison to the corresponding fractions in experiment 4 we can still see the persistence of inhibition of cleavage to the same degree or greater. Within experiments 7 and 8 also we can see for example that fractions 2, 3, 5 and 7 in experiment 8 showed stronger inhibition than the more concentrated corresponding fractions in experiment 7. These comparisons emphasize again that the time of application of the tested material and the condition of the sea urchins are primary factors in causing variations observed.

In contrast to inhibition of cleavage which was observed in experiments 1-13, stimulation of cleavage appeared in most fractions of experiments 15, 16, 18, 19, 20, 21 and 22 (Table III). One difference in the treatment of the DNA fractions here from the treatment of fractions in experiments 1-13 was the use of the method suggested by Dr. Tyler for adjusting the salinity of the fractions to that of sea water, namely by dialyzing the fractions for two days against sea water.

Table IV includes the results of two experiments, 14 and 17 which were basically similar both aiming at finding whether the inhibitory material referred to earlier could be removed by dialysis. In experiment 14 fractions 2, 3 and 4 were dialyzed against distilled water. Then the salt contents of the material in the bags and the material outside the bags was adjusted to that of sea water. Table IV

indicates that in the materials which diffused through the bags from fractions 2, 3 and 4 the eggs had 2%, 38.8% and 78% cleavage respectively compared to 100% cleavage for the control. The material which remained in the bags after dialysis of fractions 2,3, and 4 was also tested on the cleavage of the eggs. Eggs from the same female sea urchin used with the diffusible material were fertilized from the same male. Table IV shows 73%, 73% and 92% cleavage in fractions 2, 3 and 4 respectively compared to 100% cleavage for the control. The results indicate that the inhibitory material was dialyzable and lend support to the idea that the inhibitory component was derived from DNA. A diphenylamine

T A B L E IV

	Digestion No. 8 Experiment 14 Date Sept. 2		Digestion No.10 Experiment 17 Date : Sept, 8
Fraction	% cleavage outside bags	% cleavage inside bags	% cleavage outside bags
1	-	-	-
2	2%*	73%*	0%*
3	38.8%*	73%*	3%*
4	78%*	92%	35%*
5	-	-	40%*
Control	100%		70%

color test for DNA derivatives in the material outside the bags gave positive results.

Table IV also shows results for experiment 17 in which the fractions were dialyzed against sea water (Dr. Tyler's

Method). The material outside the bags was then tested on cleavage of the eggs. The cleavage percentages were 0%, 3%, 35%, and 40% for fractions 2,3,4 and 5 respectively compared to 70% cleavage for the control. Thus here we have another indication beside the indication from experiment 14, that the inhibitory material was dialyzable.

4. Cleavage In Artificial VS. Natural Sea Water

In Table III artificial sea water was used as a control for experiments 1-13 while natural sea water was used as a control for experiments 15,16,18,19,20,21 and 22. Comparison of the percentages of cleavages in the controls of the two sets of experiments shows that the percentages of cleaving eggs were higher in artificial sea water controls than in natural sea water controls. The average percentage of cleavage in the former was 71% while in the later the value was 23%. An attempt was made to get a more clear understanding of this relationship of both kinds of sea water to cleavage of the sea urchin eggs. Experiments were made in which a direct comparison could be made between cleavage in natural sea water and in artificial sea water. Both kinds of sea water were tested on cleavage. The results are reported in Table V and indicate that no appreciable difference in effects existed between artificial and natural sea waters. These results give some indication that the observed differences among the two sets of controls in Table III should have been influenced by the time of the year. The existence of poor cleavage of sea urchin eggs

during a certain period in summer had been observed by Butros (1959). Besides in this study the control eggs in natural sea water after starting cleavage were usually observed to be unable to complete it by formation of clear cut furrows as observed on eggs in the experimental fractions especially those earlier fractions from 1-4.

T A B L E V

Experiment No.	% cleavage in natural sea water	% cleavage in artificial sea water
1	75%	79%
2	67%	74%
3	70%	67%
4	87%	76%

DISCUSSION

The results obtained in this study indicate that there were two components each consisting of one or more DNA derivatives. Among the two components one was inhibitory to cleavage while the other was stimulating depending on the way in which materials were prepared and possibly the time of the year.

When the salinity of the fractions was adjusted to that of artificial sea water without dialysis, as in experiments 1-13, inhibition of cleavage appeared in several fractions and was most pronounced in fraction 2 Table III. Available evidence indicates that the inhibitory effect was caused by one or more DNA derivatives. The lines of evidence are the following:

1. Occurrence of inhibition in several fractions throughout experiments 1-13 (Table II). Though in some cases the percentages of cleaving eggs in the controls were low, the cleavage percentages in some fractions was still much lower.
2. The occurrence of highest inhibition in fraction 2 suggests that an inhibitory component was most concentrated in this fraction. This component could not be but derived from DNA. It is important to note here that fraction 2 as Table II A and B indicates was not the most concentrated fraction in total DNA derivatives.

This means that an interpretation of inhibition on the basis of a high concentration of DNA derivatives cannot be made.

3. The existence of the dialyzable inhibitory component the presence of which was demonstrated by experiments 14 and 17 where dialysis was used also indicates that the inhibitory component was derived from DNA since no other source for a dialyzable component is possible. Besides the appearance of stimulation in experiments 15, 16, 18, 19, 20, 21 and 22 where dialysis was also applied on the fractions suggests that such an inhibitory dialyzable component had escaped during dialysis.

It will be recalled that stimulation of cleavage appeared in experiments in which the salinity of the fractions was adjusted by dialysis against natural sea water. Several factors may explain the appearance of this effect. These include the following:

1. Escape of a dialyzable inhibitory component during dialysis leaving behind an effective concentration of a stimulating component consisting of one or more DNA derivatives which may be less dialyzable or non-dialyzable.
2. Occurrence of a qualitative change among the DNA derivatives brought about by the action of salts from the sea water against which the fractions were

dialyzed. Such salts have been found to degrade DNA or DNA derivatives. Zamenhoff and Chargaff (1951) found that Mg^{++} broke down apurinic acid, a product of acid hydrolysis of DNA. Moreover Tamm et al (1952) found that deoxypolynucleotides of low molecular weight were degraded by Mg^{++} and Mn^{++} .

3. A third possible explanation for the stimulating effect of some DNA derivatives on cleavage is the idea suggested by Butros (1959) that the summer eggs might have been in a deficient state which was illuviated by DNA derivatives. The fact that the eggs seemed to start cleavage and that the cleavage furrows could not go deep enough to complete cleavage suggests the possible existence of a deficiency. This deficiency in the eggs may be related to the reduced size of the ovaries which seems to take place after shedding of the eggs at the earlier part of the breeding period. Those eggs shed at this period divide favourably while eggs which are retained in the ovaries after shedding usually divide poorly.⁹ This difference could be due to a deficiency in the supply of nutrient material for the eggs retained

9 . In some cases the eggs had high percentages of cleavage (see Table III). This was possibly due to variations in the extent and time of shedding eggs for different individuals.

in the ovaries after shedding. The nutrient material may depend on a supply of DNA or DNA derivatives.* Studies by Schrader and Leuchtenberger (1952) on a species of Hemipteran insects revealed that DNA material is transferred from the nurse cells of the ovaries to the eggs where it is stored in the cytoplasm and contributes to the nutritive material of the egg. The finding reported by Brachet (1957) of DNA fragments and DNA derivatives in the cytoplasm of frog eggs and sea urchin eggs suggests that what takes place between ovaries and eggs of the Hemipteran insect may be taking place also in other animals such as the frog and sea urchin. If the supply of nutrient material does not reach the eggs in time (presumably before the shedding season) the eggs tend to have poor cleavage.

In conclusion the results agree with the results obtained by Butros (1959) with regard to the occurrence of a stimulating component among DNA derivatives obtained by degradation of highly polymerized DNA by DNA-ase. They further indicate that among such derivatives there is a component which inhibits cleavage. The chemical composition and mechanism of action of the two components is not known, but it appears that the inhibitory component consists of molecules which are of smaller size and are more dialyzable than molecules of the stimulating component.

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A D D E N D U M

During discussion of this thesis, the committee recommended this note concerning a possible explanation of the variability in inhibition and stimulation. This may have been caused by a dark colored growth on the Ecteola - cellulose column - which later proved to contain at least 3 types of Penicillia and a yellow bacterium. Washing the column with water was not sufficient to prevent this growth. It was suggested that the column be treated in a way to prevent this or to use a new column each time. Dr. Kerr suggested a 1:10,000 solution of merthiolate which he says does not interfere with enzyme action.