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Studies of Protein-bound Sulfhydryl and Disulfide Groups in the Mitotic Apparatus of the Sea Urchin, Arbacia punctulata

Naoko Kawamura
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To the Graduate Council:

I am submitting herewith a dissertation written by Naoko Kawamura entitled "Studies of Protein-bound Sulfhydryl and Disulfide Groups in the Mitotic Apparatus of the Sea Urchin, Arbacia punctulata." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Ronald C. Fraser, Major Professor

We have read this dissertation and recommend its acceptance:

Sammuel R. Tipton, David M. Prescott, Jordon Carlson, Tosh P. Solo

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

August 16, 1960

To the Graduate Council:

I am submitting here with a thesis written by Naoko Kawamura entitled "Studies of Protein-bound Sulfhydryl and Disulfide Groups in the Mitotic Apparatus of the Sea Urchin, Arbacia punctulata."
I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with major in Zoology.

Ronald C. Fraser
Major Professor

We have read this thesis and recommend its acceptance:

Samuel R. Tipton

David M. Prescott

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Dale Worthington
Dean of the Graduate School

STUDIES OF PROTEIN-BOUND SULFHYDRYL AND DISULFIDE GROUPS IN THE MITOTIC APPARATUS OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

A Dissertation

Presented to

the Graduate Council of

the University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Naoko Kawamura

August 1960

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INTRODUCTION

A. General introduction of sulfhydryl and disulfide groups

Sulfhydryl (SH) compounds are extremely reactive substances. They oxidize and reduce easily, combine with a large number of halogen-containing substances, and combine with heavy metals forming mercaptides.

SH compounds occuring in biological systems are divided into two types: the soluble SH compounds, of which glutathione is a representative example, and the fixed SH groups of the proteins. According to their reactivity with various reagents, the latter are divided into three types (Barron, 1951). Some SH groups in native protein react readily with nitroprusside and mild oxidizing agents. These are called the freely reacting SH groups. Other SH groups in native protein will react only with more powerful reagents such as iodine and the mercaptide-forming reagent (p-chloromercuribenzoic acid); they may be designated as the sluggish SH groups. Finally, there are other SH groups which remain in native protein so well protected that the known SH reagents do not attack them until molecular configuration is changed by denaturation; these are the masked SH groups.

Disulfide (S-S) groups are formed when two SH groups are oxidized with an oxidizing agent, and this reaction is easily reversed in the presence of a reducing agent.

SH compounds have been found to be very important in the activity of many enzymes, in cellular respiration, and in cell division and growth.

B. SH compounds in cell division

Since the time of the pioneer studies of Rapkine (1931), SH compounds have been assumed to play an important role in cell division. Rapkine's observations, later confirmed by Bolognari (1952), revealed that there was a significant decrease in "non-protein" SH groups at the mid-point of the division cycle of sea urchin eggs and an increase reaching a maximum shortly before the egg divided. The addition of a small amount of $HgCl_2$ to fertilized sea urchin eggs stopped mitosis completely. Rapkine postulated that the processes of cell division were dependent on an increase in reactive SH groups in the cell. Furthermore, he considered that the increase in SH groups was produced by a reversible denaturation of intracellular proteins which transformed masked and sluggish SH groups to freely reacting ones. He did not show, however, how the SH groups were involved in the mechanism of cell division.

One of the roles of SH groups in cell division was suggested, twenty years after Rapkine's remarkable experiment, by Mazia and Dan (1952), and Mazia (1954), to be in the formation of the mitotic apparatus. Mazia and Dan (1952)

succeeded in isolating mitotic apparatuses from sea urchin eggs and subjected them to biochemical analysis. They found that the isolated mitotic apparatuses were not dissolved in the solubilizing agent, Duponol D, while the mitotic apparatuses treated by sodium thioglycollate were completely dissolved in the same solution. Since sodium thioglycollate is known to be a strong reducing agent for S-S groups, these workers suggested that the fibrous structure of the mitotic apparatus consists of chains of protein units polymerized largely through intermolecular S-S bonds. Later, using Rapkine's (1931) estimation of the quantity of soluble SH groups, Mazia (1954) proposed an hypothesis for the mechanism of formation of spindle and astral fibers. According to his scheme, in the first half of the division cycle the loss of soluble SH groups is due to the linking of such substances as glutathione to protein by S-S bonding. During the phase of the formation of the mitotic apparatus when soluble SH groups increase, the small molecular SH compounds are freed from the protein with subsequent formation of S-S linkage between protein strands.

Very recently, however, Neufeld and Mazia (1957), and Sakai and Dan (1959), after careful study, have shown that non-protein SH compounds are constant in quantity throughout the first division of sea urchin eggs. Sakai and Dan (1959) have reported that Rapkine and Bolognari had measured the SH

groups of not only small molecules but also of some protein dissolved in the 25% trichloroacetic acid (TCA) used in their extraction procedure.

Protein-bound SH groups in the mitotic apparatus of sea urchin eggs have been studied cytochemically by Kawamura and Dan (1958). Using several cytochemical methods, they have demonstrated abundant SH groups in the spindle and astral fibers.

C. Problems in the present study

From the studies of Sakai and Dan (1959), it became apparent that the changes in soluble SH groups during the first division of sea urchin eggs, as reported by Rapkine (1931), represent the changes in protein SH groups dissolved in 25% TCA. It is also certain from the cytochemical study of the mitotic apparatus (Kawamura and Dan, 1958) that there are abundant SH groups in the protein of spindle and astral fibers. However, there is no evidence to show whether the total amount of protein-bound SH groups (present in both the 25% TCA-soluble and insoluble protein) changes during the course of formation of the mitotic apparatus, whether the amount of protein-bound S-S groups changes, and whether there exist S-S groups in the mitotic apparatus as stated by Mazia (1954). If there is an increase in the amount of S-S groups in fibers of the mitotic apparatus, where do they

originate? In order to answer the above questions and to uncover the secret of the mechanism of formation of mitotic apparatus, it is necessary to measure protein-bound SH groups as well as S-S groups. It is also essential to correlate cytochemical observations with the data derived from the measurements of such groups.

In the present study, changes in protein-bound SH and S-S groups in sea urchin eggs during the first division cycle were followed cytochemically and quantitatively. Special attention is given to the possible role of these groups in the structure and origin of the mitotic apparatus of sea urchin eggs.

MATERIALS AND METHODS

Material

Eggs of Arbacia punctulata were used. These were kindly supplied by the Oceanographic Institute of the Florida State University during the writer's visits there.

Eggs were spawned into sea water following M/2 KCl stimulation, inseminated with a dilute sperm suspension and fixed at the following stages: unfertilized egg, immediately after fertilization, syngamy, early streak, late streak, mitosis and the 2-cell stage (see Figure 1). Eggs were fixed with 5% TCA, because it had been shown previously (Kawamura and Dan, 1958) to be an excellent fixative for subsequent staining of SH groups. The eggs were embedded in paraffin on the day following fixation and used for the present study.

Method

A. Quantitative determination of SH and S-S groups

1. Principle of the method

The general method for quantitative measurement of SH groups is based on the following principle. A colored reagent is permitted to react with the SH groups in cells or tissues. The biological material is then removed from excess reagent, and placed in a known

amount of a suitable solvent. A reducing agent is added, under the influence of which the reaction is reversed, and the amount of colored reagent liberated into the solvent is measured spectrophotometrically.

2. Reagent

For the purpose of the present study, the reagent used must (1) have a high specificity for SH groups, (2) have a completely reversible association with SH groups, and (3) be perceptively colored. Hellerman and co-workers' extensive study (1943) on sulfhydryl reagents has shown that organic mercuric compounds have a high specificity for SH groups. The red sulfhydryl reagent (RSR), 1,4-(chloromercuriphenylazo)-naphthol-2, was synthesized by Bennett and Yphantis (1948) and has been proved to have a high specificity for SH groups by Bennett (1951) and Kawamura and Dan (1958). Reversible association of RSR to protein-bound SH groups was tested as follows: After denatured egg albumin was placed in a known concentration of RSR-acetone solution for 3 hours, the amount of the reagent taken up by the albumin was measured. The excess reagent was washed out with acetone, a known amount of acetone containing a small amount of 2,3-dimercaptopropanol (BAL) was added to the stained albumin to release RSR into acetone, and the amount of released RSR was measured. In this

test, RSR showed a completely reversible association with SH groups.

3. Solvent

The solvent used in the present study must (1) dissolve RSR sufficiently for assay in a spectrophotometer, and (2) not interfere in the reaction between SH and the reagent. The optical transmission of 10^{-4}M RSR in acetone is only 4% at the wave length of 480 millimicra, which is the maximum of the absorption spectrum of RSR. Absolute acetone is therefore considered to be a suitable solvent on the basis of solubility. In regard to the second criterion, the interaction of SH groups with RSR in the presence of various solvents was examined. Absolute acetone, buffered 50% acetone (pH 8.0, NaOH-KH_2PO_4 buffer), butanol, 80% ethanol and buffered 80% ethanol (pH 8.0, NaOH-KH_2PO_4 buffer) were used as solvents. Frog muscle and sea urchin eggs fixed with 5% TCA were embedded in paraffin and sectioned at 15 micra. After the paraffin was removed by xylol, the materials were stained in 12 milliliters centrifuge tubes with the reagent dissolved in the solvents indicated. Stained materials were dehydrated by ethanol and mounted in balsam on microscope slides. Buffered 80% ethanol was used in the staining solution of the present study, because, on the basis of uptake of stain by the biologi-

cal materials, it was considered to be superior to the other solvents tested.

4. Method for determination of protein-bound SH groups

Eggs of the appropriate stage were fixed in 5% TCA. After they had been fixed for 12 hours, the eggs were repeatedly washed with distilled water for a total of 30 minutes, passed through successively increasing concentrations of distilled ethanol to xylol and embedded in paraffin. Eggs were sectioned at 15 micra, and the paraffin was removed with xylol in a centrifuge tube. The eggs were washed with absolute ethanol, then with buffered ethanol (absolute ethanol diluted by M/40 NaOH-KH_2PO_4 buffer, pH 8.0). The staining solution was prepared by adding an excess of finely ground RSR to buffered 80% ethanol solution followed by filtration of the orange-colored liquid. Sectioned eggs were suspended in this solution and stained for 3 hours, then the excess dye was completely removed by washing with several changes of absolute acetone. The stained eggs were divided into two groups; one to be used for a quantitative estimation of SH groups, and the remainder reserved for cytochemical observation (see section B for this procedure). The eggs stained for the quantitative measurement of SH groups were placed in 12 milliliters centrifuge tubes with about 9 milliliters of

absolute acetone, and the liberation of the dye was brought about at 50°C by the addition of a few drops of diluted BAL, until all coloration in the eggs had disappeared. After reduction, which cleaves the azo-dye from the egg protein SH groups, the suspension of the sectioned eggs was adjusted to 10 milliliters with absolute acetone, centrifuged, and accurately readjusted to 10 milliliters with a few drops of the solvent. Optical transmission of the colored supernatant fluid was measured in a Beckman spectrophotometer at a wave length of 480 millimicra. All solvents used were redistilled, in order to eliminate any contaminations with heavy metals, a minute amount of which can act catalytically in the oxidation of SH groups to S-S groups. Protein nitrogen of the eggs was determined by the microKjeldahl-Nesslerization method. The precision of this method in the determination of the SH concentration in frog muscle protein under these condition is shown in Table 1. Since the largest standard error (SE) obtained is 0.0665, which represents only 2.3% of total amount of SH groups measured, it is considered that the precision of the method used is adequate for the purpose of the present study.

5. Method for S-S determination

In the determination of protein-bound S-S groups,

TABLE I

THE PRECISION OF THE METHOD USED FOR THE DETERMINATION OF SULFHYDRYL GROUPS IN THE FROG MUSCLE

Frog No.	Number of Tests	Moles SH x 10^{-10}/g protein N.	
		Mean ± S.E.	Range
1	5	2.804 ± 0.0665	2.62-3.03
1	6	2.747 ± 0.0185	2.68-2.82
1	5	3.148 ± 0.0638	3.00-3.31
2	6	3.187 ± 0.0210	3.11-3.26
2	6	3.433 ± 0.0655	3.20-3.61
3	6	3.425 ± 0.0647	3.25-3.60
3	6	3.437 ± 0.0358	3.32-3.58

0.5M BAL in absolute ethanol containing 0.4% KOH was used as a reducing agent according to the method of Teiger, Farah and DiStefano (1957). After reduction of the S-S groups, BAL was carefully washed out with several changes of buffered 80% ethanol and then with the staining solution, until the color of the dying solution remained dark, and the eggs were deeply stained. A determination of SH groups, as outlined above, was then carried out in reduced eggs in order to obtain the amount of S-S groups.

B. Cytochemical method for protein-bound SH and S-S groups

Cytochemical preparations were made of the remainder of the stained eggs mentioned above in the section A-4. Observations were carried out, after the stained eggs had been cleared in xylol and mounted in balsam on microscope slides, with the use of a blue filter made from photographic film stained by toluidine blue (Kawamura and Dan, 1958).

C. Cytochemical method for protein

The morphological changes in the mitotic apparatus after reduction of S-S groups were examined with the staining method for protein developed by Mazia, Brewer and Alfert (1953). The paraffin of sectioned eggs was removed in centrifuge tubes, then the egg sections were hydrated through

successively decreasing concentrations of ethanol and stained with a solution containing 10 grams of $HgCl_2$ and 100 milligrams of bromphenol blue per 100 milliliters of water. Then the eggs were washed for one minute in 0.5% acetic acid and for 15 minutes in tap water. After quick dehydration, the stained eggs were mounted in balsam on microscope slides.

D. Method of isolation of mitotic apparatus

Isolation of the mitotic apparatus of Arbacia eggs was carried out according to the modification by Mazia (1954) of the procedure of Mazia and Dan (1952). The eggs were treated for 4 minutes in a 0.01% solution of crude protease in sea water to prevent formation of the fertilization membrane, washed in sea water, and fertilized. When the eggs had reached the mitotic stage, they were fixed with 30% redistilled ethanol at -10°C. After at least three days in this medium at -10°C, the eggs were added to 1% solution of digitonin and shaken to disperse the cytoplasm. In addition to 30% ethanol, 30% ethanol containing M/1000 ethylene diamine tetraacetic acid (versene) was used as fixative. The isolated mitotic apparatuses were observed with a phase contrast microscope for their fibrous structure.

RESULTS

A. Changes in amount of SH and S-S groups

Changes in the amount of protein-bound SH groups of *Arbacia* eggs at various stages after fertilization are shown in Figure 1. Because the time interval from insemination to cleavage varied from 55 minutes to 80 minutes in different batches of eggs, the cleavage time was adjusted to 60 minutes in the graph (Figure 1, 2, 3). Corresponding corrections were made for other stages of development so that in all groups a similar stage is represented in all eggs at one given time. The SH content in unfertilized eggs varied from 3.4×10^{-10} to 5.8×10^{-10} moles SH/γN., with an average of 4.44×10^{-10} moles. In Figure 1, the SH concentrations are expressed as the percentage of the SH content of unfertilized eggs.

Figure 2 shows changes in amount of protein SH plus S-S groups. The mean concentration of SH plus S-S groups in the unfertilized eggs is 6.38×10^{-10} moles/γN.

The means of the SH or SH plus S-S determinations at various stages have been statistically analysed for differences at the 5% level according to Duncan's multiple range test (1955).

There is, as shown in Figure 1, a statistically significant lowering of SH content at the early streak stage (26

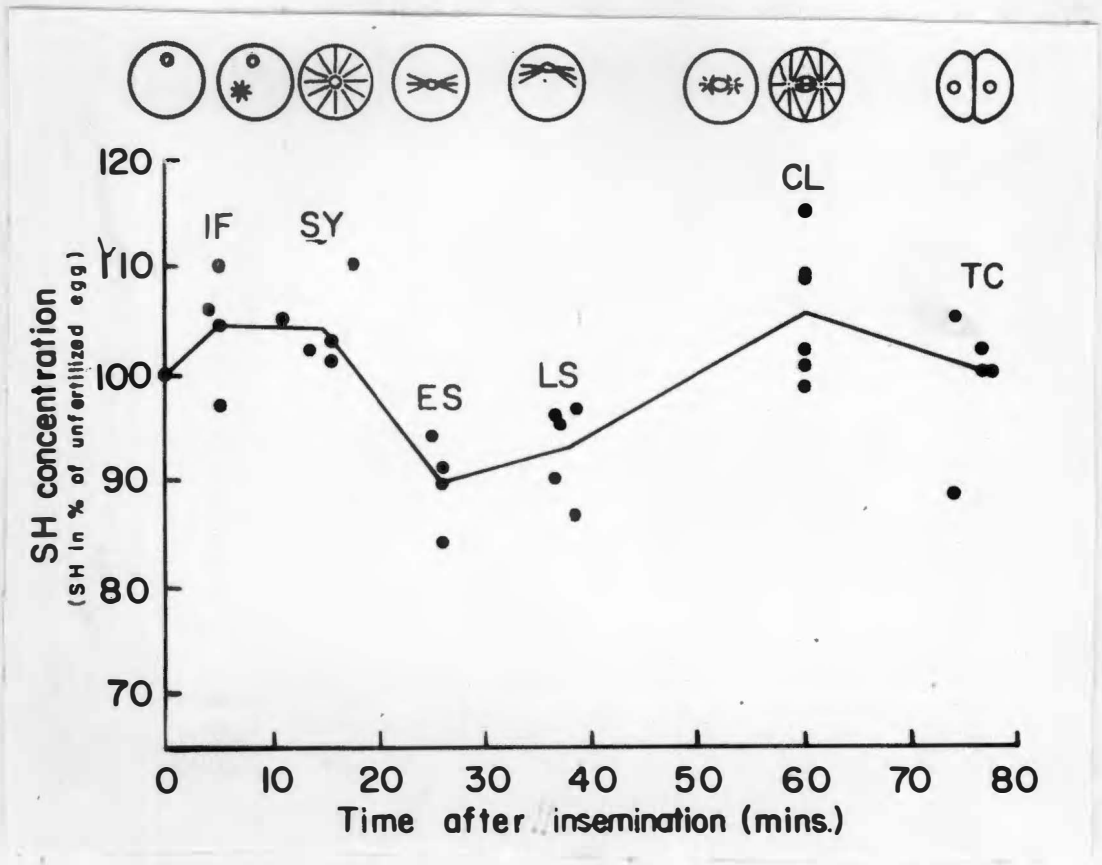

Fig. 1. Concentration of protein SH groups during the first cleavage of Arbacia punctulata. SH content is shown as the percentage of that of the unfertilized egg. The only statistically significant changes in the concentration of protein SH groups are a decrease at the early streak stage and a rise between late streak and cleavage stages. IF:immediately after fertilization; SY:syngamy; ES:early streak; LS:late streak; CL:cleavage; TC:2-cell stage.

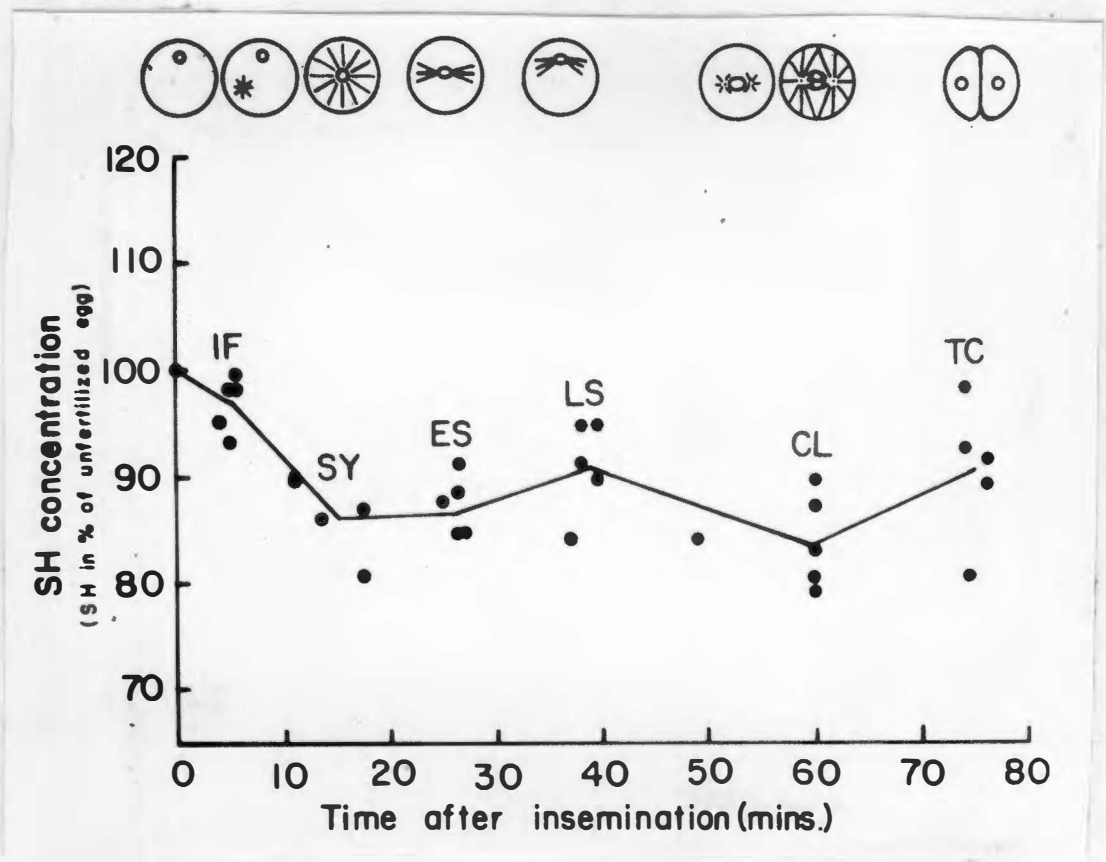

Fig. 2. Concentration of protein SH plus S-S groups during the first cleavage of Arbacia punctulata. SH concentration is expressed as a percentage of that present in the unfertilized egg. There is a fall in protein SH plus S-S groups from fertilization to syngamy stage, thereafter, no significant change in concentration of these groups. IF:immediately after fertilization; SY:syngamy; ES:early streak; LS:late streak; CL:cleavage; TC:2-cell stage.

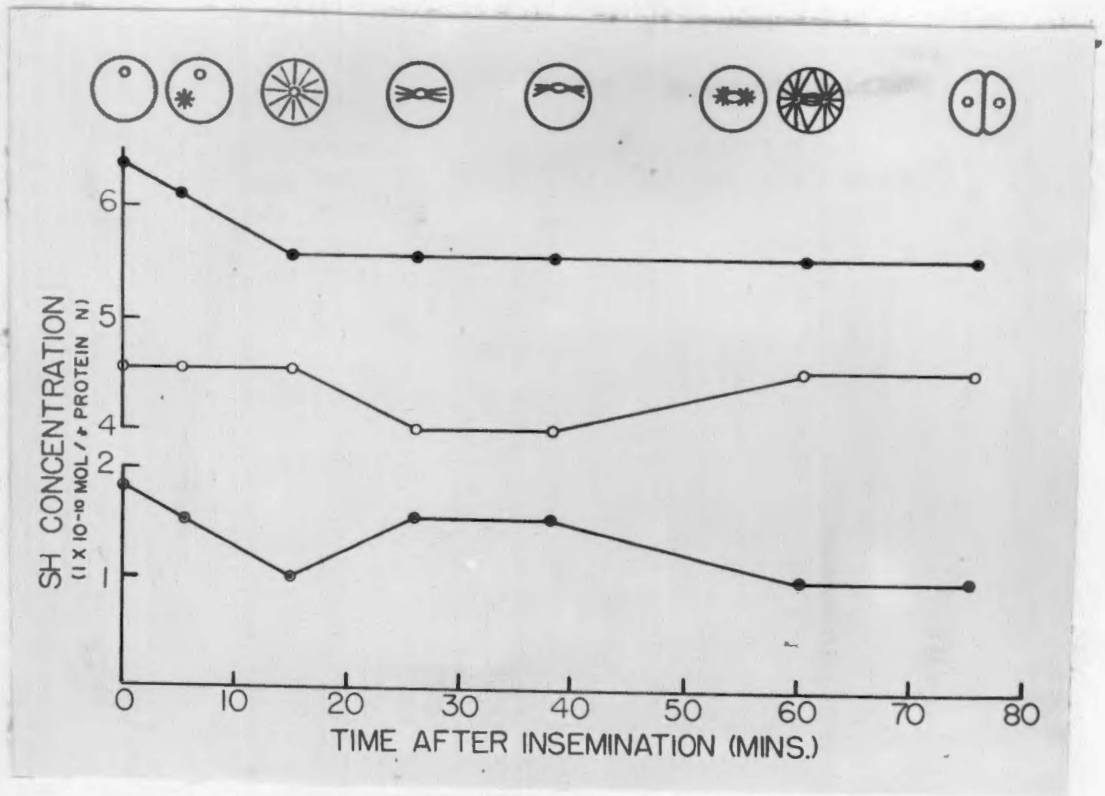

Fig. 3. Changes in SH groups (open circle) and SH plus S-S groups (solid circle) during the first cleavage of Arbacia eggs. S-S groups alone (dotted circle) are obtained by subtraction of SH groups from SH plus S-S groups.

minutes after fertilization) and a significant rise at the cleavage stage (60 minutes after fertilization). No significant difference, however, could be found in the concentration of protein SH groups in the unfertilized egg (0 minute), immediately after fertilization (5 minutes), syngamy (17 minutes), cleavage (60 minutes) and 2-cell stage (74 minutes).

As revealed in Figure 2, there is a significant decrease in the sum of protein SH and S-S groups appearing at the syngamy stage (17 minutes), then no further significant changes occur. These results are diagramatically summarized in Figure 3.

B. Changes in distribution of SH and S-S groups

SH groups: The changes in the distribution of protein-bound SH groups during the first cleavage are almost the same as those obtained previously in four different species of echinoderms, Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Mespilia globulus and Clypeaster japonicus (Kawamura and Dan, 1958). The nuclei of Arbacia eggs were not deeply stained at any stage (Fig.4:1A~6A, Fig.5:12A). As diasters were formed, deeply stained astral centers appeared at the poles of the ovoid-shaped nucleus (Fig.6:7A). Before the nuclear membrane disappeared, deeply stained fibers appeared between the two astral centers (Fig.6:13A). The spindle, astral centers and chromosomes stained deeply

throughout the prometaphase to anaphase (Fig.5:8A~10A). The disappearing astral centers and spindle stained faintly during telophase (Fig.5:11A). In the 2-cell stage, the newly formed nuclei were unstained like those in the earlier stages (Fig.5:12A).

SH plus S-S groups: After reduction of S-S groups with BAL prior to staining with RSR, the egg nuclei were still not deeply stained at any stage of development (Fig.4:1B~6B, Fig.5:12B). The egg cytoplasm of pre-diastral embryos, however, stained uniformly deeper after reduction of the S-S groups than they did before reduction. It is notable that the astral centers, spindle and centrosphere were stained to the same extent as those stained for pre-existing SH groups alone. However, the remaining region of the cytoplasm appeared more deeply colored than in eggs stained without reduction by BAL. There thus appears an hour-glass shaped centrosphere in the deeply stained cytoplasm (Fig.5:7B~11B). Observation under high magnification revealed no significant changes in stainability or appearance of asters, spindle or chromosomes before and after reduction of S-S groups (Fig.6: 13A~15A, 13B~15B). These figures show that the S-S groups are richly present in the cytoplasm of Arbacia eggs, but not in the nucleus, centrosphere, astral centers and spindle.

C. Morphological demonstration of the mitotic apparatus

before and after reduction of S-S groups

Eggs were stained for protein, according to the method described by Mazia, Brewer and Alfert (1953), both before and after reduction of S-S groups with BAL. No difference in the appearance of the spindle and astral fibers could be observed between eggs treated and eggs untreated with the reducing agent (Fig.6:16A, 17A, 16B, 17B).

D. Dissolution experiment on the isolated mitotic apparatus in alkaline solution

Mitotic apparatuses of Arbacia eggs were isolated according to Mazia's technique (1954), and subjected to treatment with alkaline solutions at pH 10.4, 11.05, 11.5 and 11.95 (prepared with NaOH), both in the presence and absence of 1% sodium thioglycollate. When the mitotic apparatus from the eggs fixed by 30% redistilled ethanol were placed in the solution at pH 11.5 either in the presence or absence of sodium thioglycollate, the fibrous structure of the spindle and asters became progressively less discernible until these structures disappeared completely. In every solution under pH 11.05, however, the isolated mitotic apparatus still showed its fibrous structure. This experiment demonstrates that there are no S-S groups in the isolated mitotic apparatus. It was impossible to isolate the mitotic apparatus, using the same technique, in the eggs fixed by 30% ethanol contain-

ing M/1000 versene.

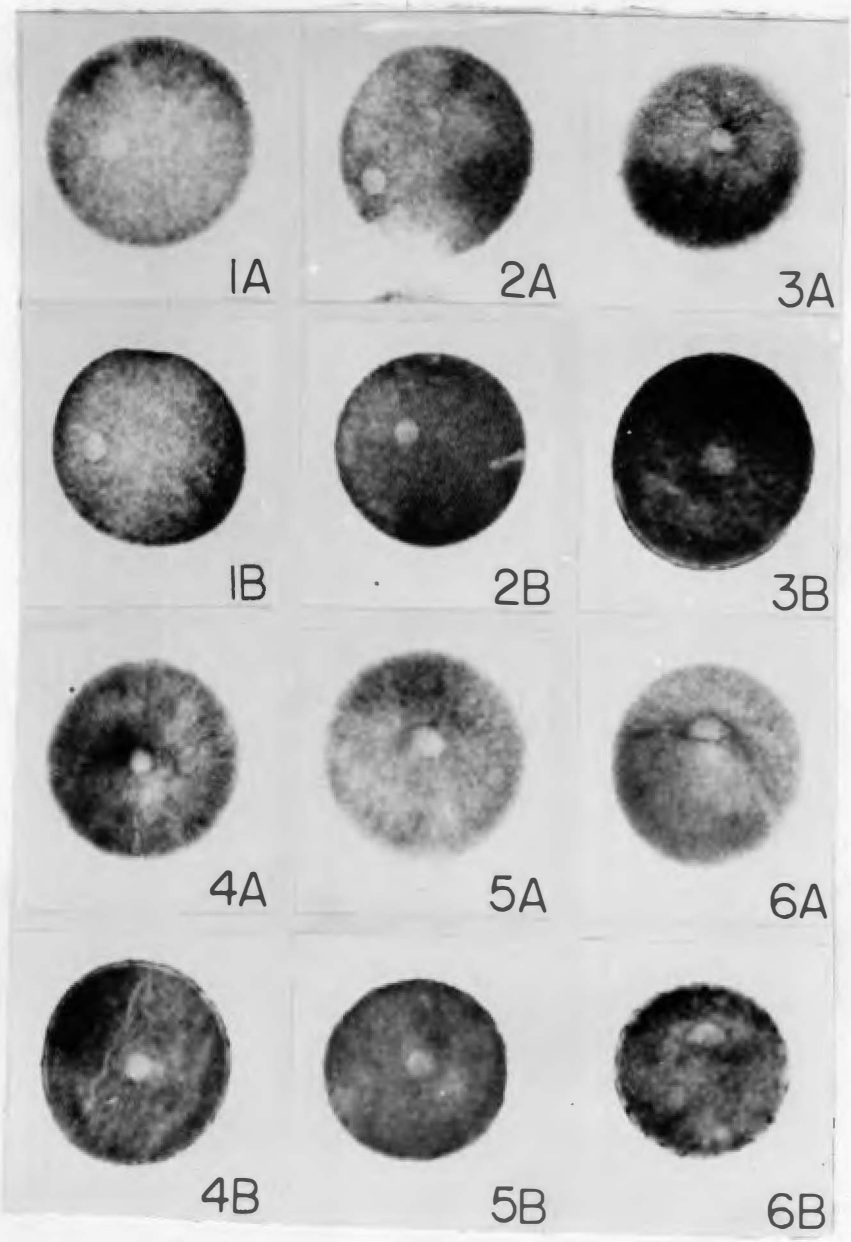

Fig. 4. Eggs of Arbacia punctulata fixed by 5% TCA and stained with RSR. A-series were stained without reduction of S-S groups. B-series were stained after reduction of S-S groups by BAL. 1:unfertilized egg. 2:immediately after fertilization (5 mins.). 3:11 mins. after fertilization. 4: syngamy (17 mins.). 5 and 6:streak stage (26 and 40 mins.).

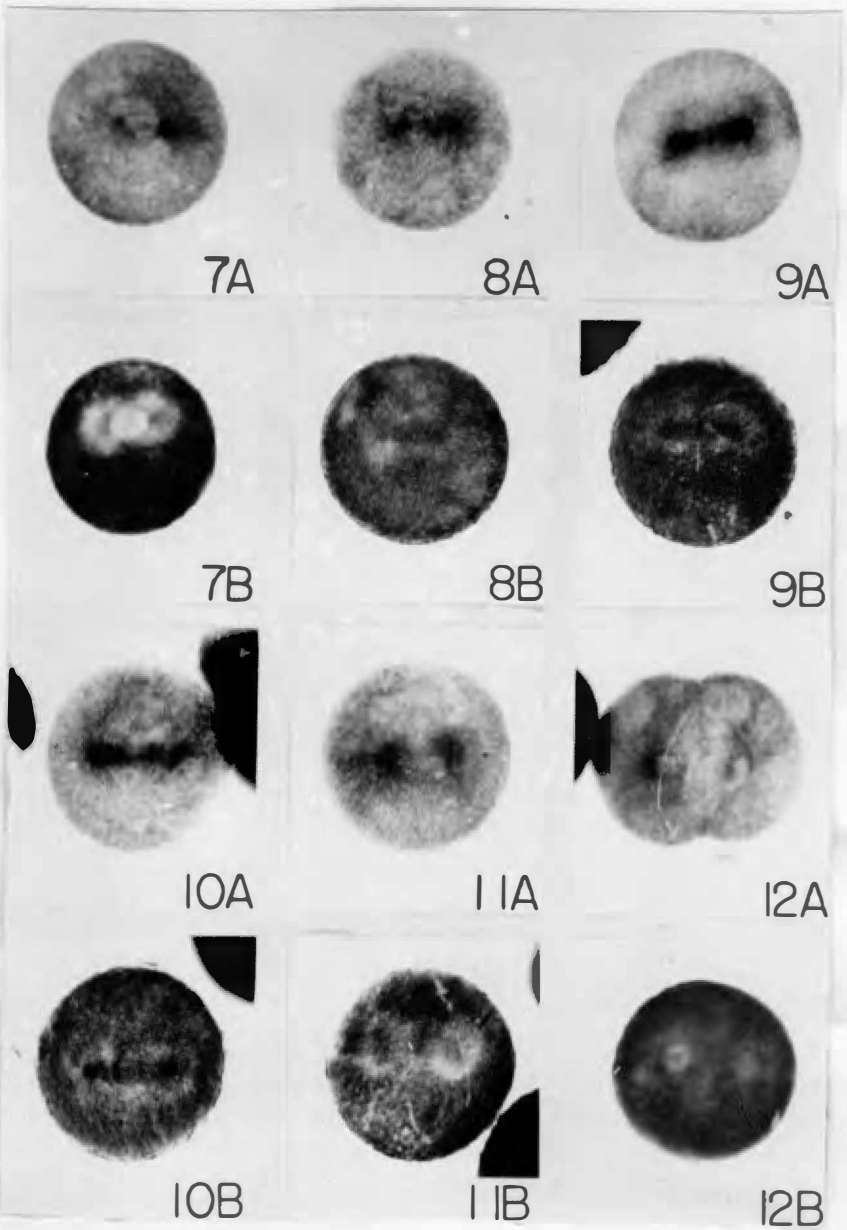

Fig. 5. A-series were stained for SH groups without reduction of S-S groups. B-series were stained after reduction of S-S groups by BAL. 7:prophase. 8:prometaphase. 9:metaphase. 10:anaphase. 11:telophase. (The stages shown in 7~11 were found in the eggs fixed at 60 mins. after fertilization.) 12:2-cell stage (74 mins.).

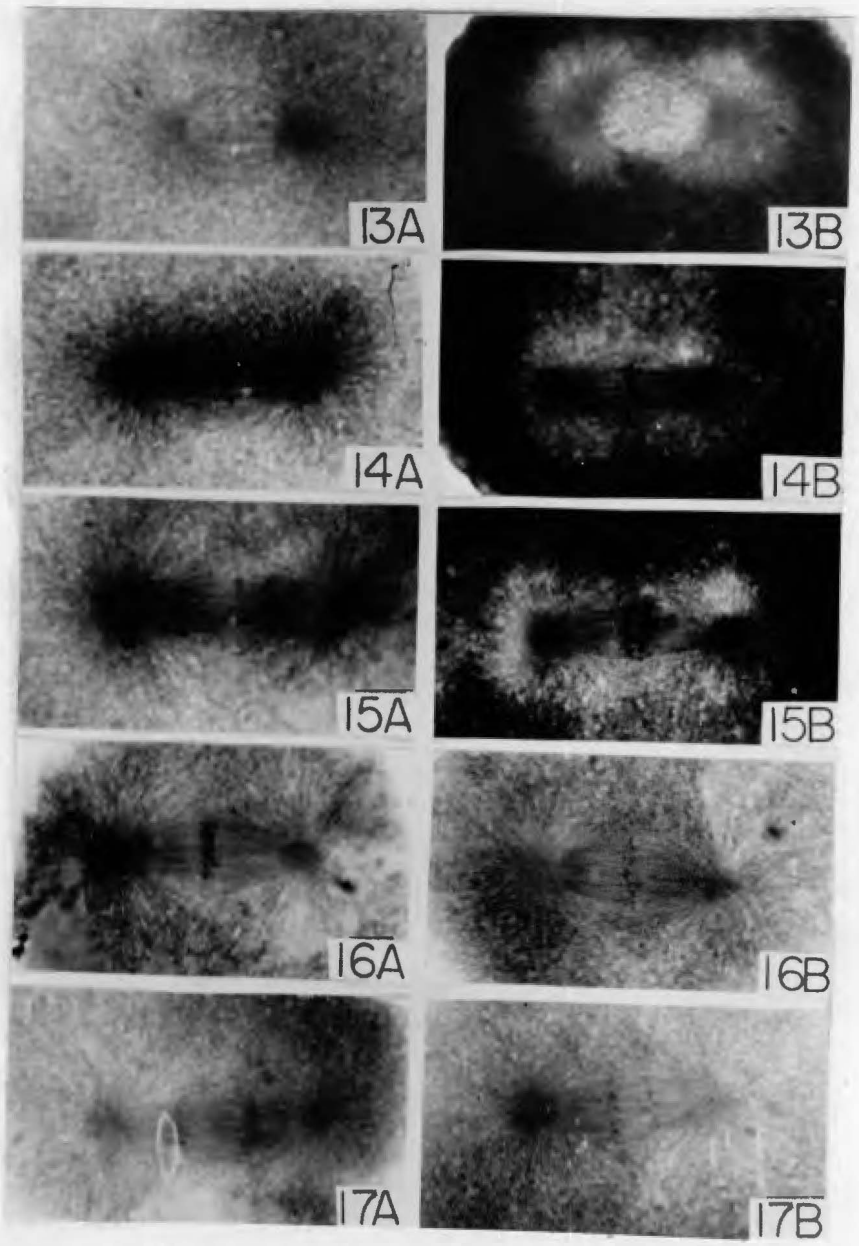

Fig. 6. A-series were not treated by BAL. B-series were treated by BAL. 13, 14 and 15 were stained with RSR. 16 and 17 were stained with bromphenol blue for protein. 13: prophase. 14:metaphase. 15:anaphase. 16:metaphase. 17: anaphase.

DISCUSSION

Recently, Neufeld and Mazia (1957), and Sakai and Dan (1959) have demonstrated that the quantity of non-protein SH compounds in early developmental stages of certain echinoderms remains constant. Moreover, Sakai and Dan have shown that the supernatant fluid from a 25% TCA-treated egg homogenate, as prepared by Rapkine's method (1931), contained not only non-protein SH compounds but also protein SH. Therefore, the fluctuations in the amount of SH groups detected by Rapkine also represent fluctuations in the 25% TCA-soluble SH protein.

As shown in Figures 1 and 3, the amount of protein-bound SH groups as determined by the method used in this study decreases at the early streak stage, and rises to its original level at the cleavage stage. These figures appear much like that given by Rapkine (1931), although it is found that the depression characteristic of the streak stage persists for a longer period than revealed in Rapkine's figure. This minimum in protein-bound SH groups occurs at the streak stage, which lasts for a relatively long period in Arbacia eggs.

Stern (1958) has reported that during diakinesis and metaphase of microsporocyte meiosis in Trillium anthers a decrease in protein-bound SH groups occurs along with a fall

in soluble SH and a rise in soluble S-S groups. These results conflict with those obtained in the present investigation. This conflict may be due to the difference in material. There are no changes in soluble SH groups and S-S groups during mitosis in sea urchin eggs (Sakai and Dan, 1959).

The amount of protein-bound S-S groups alone is obtained by subtraction of SH group from SH plus S-S group values. Since the amount of SH plus S-S groups does not change, except during the first 15 minutes, we must assume that S-S groups increase at streak stage at the expense of SH groups, and decrease by the reverse path during cleavage, when the asters and spindle develop fully. Cytochemical observation showed that there can be only an insignificant amount of S-S groups in the centrosphere, asters and spindle. Because the stainability and form of the mitotic apparatus do not change before and after reduction of S-S groups (Fig. 6:13A~17A, 13B~17B), we can hardly imagine that some protein in the mitotic apparatus is lost by cleavage of S-S bonds.

When we consider simultaneously the results from both cytochemical and quantitative studies, it seems possible that a part of the fibers of the mitotic apparatus is composed of SH proteins which arise from S-S proteins in the egg cytoplasm, especially from the centrosphere. There are two experiments which support this consideration. From a

microdissection study of sea urchin eggs, Lorch (1952) has demonstrated that the materials for the asters are located in the centrosphere. When she removed the nucleus from a blastomere of the sea urchin egg, asters could be regenerated in succeeding division cycles as long as the centrospheres were left intact. On the other hand, if the hyaline cytoplasm of centrospheres was removed a considerablly long lag period appeared before centrospheres were regenerated, and only after this, asters could be formed. Kawamura and Dan (1958) have observed that hyaline spots, rich in SH protein, appeared in the egg cytoplasm in place of the well developed centrosphere, when the development of asters was suppressed by ether treatment.

In their first experiment of the isolation of the mitotic apparatus, Mazia and Dan (1952) used concentrated H_2O_2 as a stabilizer to protect the mitotic apparatus from dissolution in Duponol D. The mitotic apparatus isolated by this method was not dissolved in 1~2% Duponol D, whereas it was dissolved completely in 1% Duponol containing sodium thioglycollate. Because there are abundant SH groups on the mitotic apparatus, it seems likely that the S-S groups described by Mazia and Dan are not originally present in the mitotic apparatus, but are formed secondarily by oxidation with concentrated H_2O_2 during the isolation procedure. Later, using digitonin, Mazia (1954) succeeded in isolating

the mitotic apparatus of the Strongylocentrotus egg without stabilization with H_2O_2. He reported that the isolated mitotic apparatus was still resistant to alkaline solutions of pH 11.5, in which the thioglycollate-treated mitotic apparatus would dissolve. This experiment showed the presence of S-S bonds in the mitotic apparatus even when it was isolated without the use of an oxidizing agent such as H_2O_2. Because the results derived from the present study did not indicate any discernible quantity of S-S groups in the spindle and astral fibers, Mazia's experiment was repeated in Arbacia eggs. In the present study, the mitotic apparatuses of Arbacia eggs isolated according to Mazia's technique (1954), were tested for their solubility in alkaline solution. When the mitotic apparatuses from the eggs fixed by 30% redistilled ethanol were placed in the solution at pH 11.5 either in the presence or absence of sodium thioglycollate, the fibrous structure of the spindle and asters disappeared completely. This result was later confirmed in Mazia's laboratory with Strongylocentrotus eggs (private communication). Zimmerman (1958) has reported that the mitotic apparatus could be dissolved at pH 9, when it was treated by p-chloromercuribenzoate or mersaryl (Salyrgan) immediately after isolation by the digitonin-method. If the isolated mitotic apparatus was previously oxidized, these reagents were no longer effective. Although he has assumed

that these reagents would split S-S bonds in the mitotic apparatus, it seems more likely that their action is by preventing the formation of S-S bonds by blocking SH groups before oxidation.

There may be S-S bonds in the mitotic apparatus, but the results derived from the present study would indicate that it is questionable whether S-S bonds play a main role in the formation of the spindle and astral fibers. It is not required that SH groups of the mitotic apparatus take the form of S-S bonding to polymerize the unit proteins into fibrous structure. Some alternative bonding involving SH groups, which is as yet unclear, must be considered.

The importance of the SH groups in the linkage of protein molecules has been demonstrated by Madsen and Gurd (1956) and Bárány (1956). The former authors found that polymerization of phosphorylase a monomer to the tetramer occurred when the enzyme, with its SH groups covered with p-chloromercuribenzoic acid, was treated with cysteine. The SH groups freed from the p-chloromercuribenzoic acid by the cysteine permitted polymerization. Bárány has studied depolymerization of actin by Salyrgan, urea and formaldehyde, and assumed that the hydrogen bond between SH and NH_2 groups plays primary role in the actin-actin bondings.

Mazia (1959) has found that when the mitotic apparatus was isolated by his most recent method, without the use of

either alcohol or detergents, it was readily soluble in neutral salt solution, but became extremely stable by exposure to calcium ions. Failure to isolate the mitotic apparatus treated by versene may be due to the calcium-binding capacity of this agent. However, by its chelation of heavy metals versene may also be effective by protecting SH groups from oxidation.

From Figure 3, it may be seen that there is a pronounced decrease in protein-bound S-S groups following fertilization until the time of syngamy when such groups become stabilized in concentration. Similar marked changes, followed by a period of relative stability, have been observed for oxygen consumption, glycogen utilization and ammonia metabolism in sea urchin eggs (Brachet, 1950). Although the disappearance of S-S groups may be tied in with some other metabolic event, there is, as yet, no clear evidence for such a relationship.

SUMMARY

1. By a new method with colored sulfhydryl reagent, 1,4-(chloromercuriphenylazo)-naphthol-2, protein-bound SH and S-S groups in the egg of Arbacia punctulata during the first cleavage were studied cytochemically and quantitatively.

2. The amount of SH groups decreased after the syngamy stage and then increased up to the original level at the cleavage stage.

3. Changes in the amount of S-S groups were the reverse of SH groups; that is, the amount increased at the streak stage and decreased at the cleavage stage.

4. Cytochemical observations showed that before the first cleavage SH groups were distributed in the cytoplasm uniformly but not in the nucleus. At the cleavage stage the spindle and astral fibers were rich in SH groups. S-S groups were present in eggs except for the nucleus at all stages and for the centrosphere at the mitotic stage.

5. The results suggest that the spindle and astral fibers are composed of proteins rich in SH groups which arise from S-S protein in the region of the centrosphere. They fail to support the hypothesis that oxidation of SH groups promotes the formation of spindle and astral fibers rich in S-S groups, and that the disappearance of these fibers is accompanied by transformation from S-S to SH groups.

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