STUDIES ON SULFHYDRYL GROUPS DURING CELL DIVISION OF SEA URCHIN EGG

III. —SH Groups of KCl-Soluble Proteins and Their Change during Cleavage

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ABSTRACT

Sea urchin egg proteins extracted with KCl are mostly TCA-soluble and, conversely, those extracted with TCA are KCl-soluble. Both groups are water-insoluble and show fluctuations in —SH content during the division cycle. The fluctuation of the —SH groups of the KCl-soluble protein of the whole egg is due to a —SH $\rightleftarrows$ —S—S— interchange within the freely reacting groups and not within the sluggish and masked —SH groups of the protein. The —SH content of the KCl-soluble protein of the egg cortex also fluctuates in a similar way.

As is well known, the cell division of the sea urchin egg is accompanied by internal changes such as the separation of the centrioles, the formation of the asters and the spindle, which result in an alteration in the cell form, eventually followed by separation of the blastomeres with the disappearance of the mitotic apparatus.

It was reported previously (13) that changes in the amount of —SH groups found in TCA extracts of dividing sea urchin eggs are due to fluctuations in the amount of TCA-soluble protein —SH rather than of glutathione, as has frequently been emphasized (3, 7, 11). The fact that —SH groups are involved in gelation (2, 5) and denaturation of protein (10) suggests that the morphological changes associated with division may be brought about by a shift of the equilibrium between sulfhydryl and disulfide groups of the proteins.

In the present paper, a —SH containing KCl-soluble fraction of the sea urchin egg is studied as an example of structural protein, and it is compared with a TCA-soluble protein. In the course of the study, it has been found that this KCl-soluble protein can be made into a thread capable of contracting. Although the report on the contractility of the model will be retained until the next paper, this fact increases the importance of the investigation of —SH groups of this fraction, particularly in connection with cell division.

MATERIALS AND METHODS

The work was done at the Misaki Marine Biological Station of Tokyo University, where the sea urchins, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, *Pseudocentrotus depressus*, and the heart urchin, *Clypeaster japonicus* are available.

The eggs were collected by KCl-induced spawning, inseminated and reared by the standard method reported in the previous paper (13).

The TCA-soluble protein fraction was prepared as was reported before (13).

Extraction of the KCl-Soluble Fraction: Eggs were washed once with chilled distilled water and at once homogenized in the cold water about ten times the volume of the eggs with a glass homogenizer. The homogenate was left standing for 3 hours to remove the water-soluble fraction and then centrifuged at 15,000 *g* for 10 minutes under refrigeration. After washing the sediment with distilled water, it was homogenized once more in cold 0.6 M KCl, kept for 3 hours longer in the cold with occasional

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TABLE I

Reduction by BAL of Pre-existing and Newly Formed —S—S— Bonds of Ovalbumin

Prep. no.	Medium for oxidation of —SH	Cysteine %, after oxidation	Medium for reduction of —S—S—	Cysteine, % after reduction
I	Non-oxidized	0.94	0.01 M BAL in mixture of acetone and KH_2PO_4-NaOH buffer	1.17
		0.93		1.21
II		0.93		1.30
		0.95		1.27
III		0.96		1.18
		0.98		—
I	0.01 M Na-tetrathionate in 0.2 M KH_2PO_4-NaOH buffer at pH 7.0	0.00	BAL	1.11
		0.00		1.10
II		0.00		1.19
		0.00		1.22
I	0.01 M cystine in 0.2 M KH_2PO_4-NaOH buffer at pH 7.0	0.00	BAL	1.24
		0.00		1.12
II		0.00		1.22
		0.00		1.24
I	1% H_2O_2 in 0.2 M KH_2PO_4-NaOH buffer at pH 7.0	0.01	BAL	1.17
		0.00		1.18
I	0.001 M *o*-iodosobenzoate in 0.2 M KH_2PO_4-NaOH buffer at pH 9.0	0.00	BAL	1.05
		0.00		1.00

stirring, and centrifuged at 24,000 *g* for 15 minutes. The transparent supernate will be called the "KCl-soluble fraction" in the following pages.

When necessity arose, the egg cortex was isolated by the method previously described (12). The micro-Kjeldahl determination was applied when necessary.

Measurement of Protein-Bound —SH Groups: The determination of the amount of —SH was performed by the method reported previously (12).

Measurement of Protein-Bound —S—S— Groups: The amount of protein disulfide was calculated by the difference between the total —SH measured after a complete reduction of —S—S— bonds, and the amount of —SH before reduction. For the purpose of reducing —S—S— bridges, 0.02 M BAL in a mixture of equal volumes of acetone and 0.1 M KH_2PO_4-NaOH buffer (pH 7.0) was made to act on the protein for 6 hours at 37°C. The data presented in Table I give assurance that complete reduction by BAL was achieved.

RESULTS

1. *Bound —SH Groups in TCA-Soluble Egg Protein Precipitated with HCl-Acetone*

As was pointed out in the introduction, the fluctuation in —SH amount in the TCA supernate of an egg homogenate is due to a fluctuation in the protein-bound —SH groups, and not to a change in glutathione as has been thought (3, 6, 7, 11). Consequently, the question arises as to whether the change in —SH amount is due to a difference in the amount of protein extractable by TCA or to a net increase in —SH content in the same quantity of protein. To obtain this information, protein-N should be measured in addition to —SH and their ratio determined. The values obtained will be designated as SH/N-TCA egg in contrast to the former determination which will be expressed as SH/egg (13).

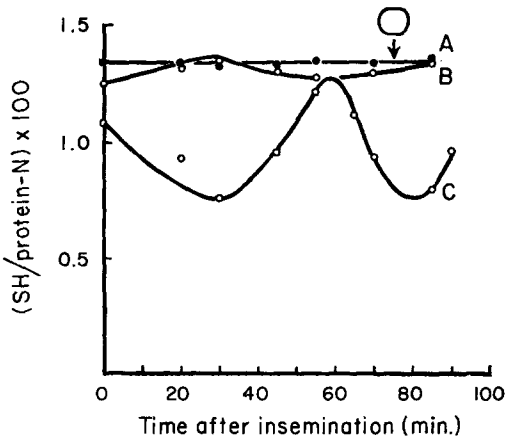

FIGURE 1

Change in (SH/protein-N) × 100 of TCA-soluble, TCA-insoluble and total protein of *Pseudocentrotus* eggs during development. A, total; B, TCA-insoluble; C, TCA-soluble.

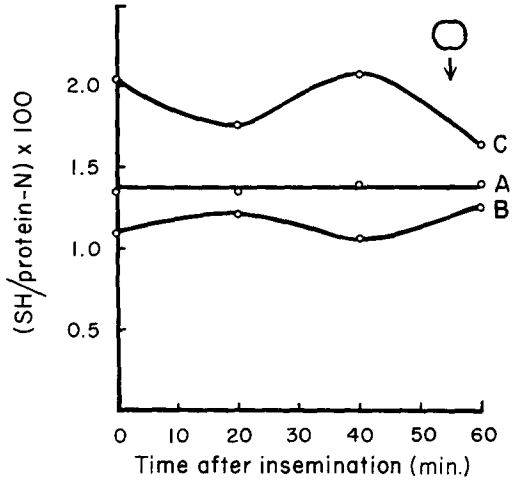

FIGURE 2

Change in (SH/protein-N) × 100 of TCA-soluble, TCA-insoluble and total protein of *Clypeaster* eggs during development. A, total; B, TCA-insoluble; C, TCA-soluble.

One volume of the TCA extract was added to 9 volumes of HCl-acetone to precipitate the proteins, and —SH groups were measured on the basis of the weight per cent of protein-N.

Before measuring the —SH of TCA-soluble and -insoluble fractions, SH/N of the total protein of the egg precipitable by HCl-acetone was followed through the early development (Curve A of Figs. 1 and 2). In both *Pseudocentrotus* and *Clypeaster*, the SH/N values of the total precipitate by HCl-acetone remain constant up to the 2-cell stage.

The SH/N of the TCA-soluble protein (SH/N-TCA egg) fluctuates in the same way as —SH of TCA extract previously obtained (SH/egg), a single difference being a slight shift of the position of the peak (Curve C of Figs. 1 and 2). The SH/N of the TCA-insoluble residue changes in a mirror image fashion to that of the soluble fraction (Curve B of Figs. 1 and 2), confirming the data of Ellis in Mazia's laboratory (7).

2. *Relation between TCA-Soluble and KCl-Soluble Fractions*

When the TCA extract is dialyzed against 0.6 M KCl after neutralization by NaOH, the protein still remains in solution. On dialyzing against distilled water, however, the protein precipitates completely. Since a protein fraction which is soluble in water from the beginning is almost completely precipitated by TCA, the TCA-soluble protein must originate from the water-insoluble residue. Since proteins of the KCl-soluble fraction are also insoluble in water but mostly soluble in TCA, the TCA-soluble and the KCl-soluble proteins share similarities, although the amount of TCA-soluble protein-N is only half that of the KCl-soluble protein-N. Consequently, in the following section, the distribution of the KCl-

TABLE II

(KCl-Soluble Protein-N/Protein-N of Egg) × 100 during Development

Time after insemination (min.)	Species		
	Pseudocentrotus	*Anthocidaris*	*Hemicentrotus*
Unf.	13.1 ± 2.6	11.5 ± 2.6	11.4 ± 1.7
25		10.6 ± 0.7	
28			10.3 ± 1.5
30	10.4 ± 0.9		
42		10.3 ± 0.7	
55	10.7 ± 1.2		
56			10.8 ± 1.6
60		10.9 ± 0.6	
65	10.5 ± 0.6		10.8 ± 1.8
75*	10.8 ± 0.7	10.2 ± 0.7	
85	10.7 ± 0.4		10.8 ± 1.6
93		10.0 ± 0.5	
103			10.0 ± 1.9

* Onset of cytokinesis.

soluble fraction and its —SH ⇌ —S—S— interchange will be considered in place of those of the TCA-soluble fraction.

3. *Distribution of KCl-Soluble Protein in the Egg*

Protein-N constitutes 15 per cent by weight of the precipitate of the KCl fraction by acetone indicating that proteins are the major component of the KCl fraction.

The N-value of the KCl-soluble protein (N-KCl egg) is 10 per cent of the whole N-value of the egg protein in three sea urchin species. It decreases somewhat after fertilization and thereafter remains almost constant until the 2-cell stage (Table II).

To determine the distribution of this KCl-soluble protein, the egg cortices and the endoplasmic granules were separated, and the protein was extracted from each after the removal of the water-soluble fraction. The ratio of the KCl-soluble protein-N of the cortices (N-KCl cortex) and of the granules (N-KCl granule) is 3:7 in unfertilized eggs and 4:6 in fertilized eggs. In the second paper of this series, the increase in the amount of cortical material with development has been reported (12). In the present work, N-KCl cortex and total protein-N (N cortex) of the cortical hull are found to increase hand in hand; as a result, the ratio N-KCl egg:N cortex stays practically constant from fertilization until the 2-cell stage (see Table III).

TABLE III

Increase in Total Protein-N and KCl-Soluble Protein-N of Hemicentrotus Egg Cortex after Fertilization

Time after insemination, min.	Total protein-N, mg. (A)	KCl-soluble protein-N, mg. (B)	B/A × 100
Unf.	0.512	0.174	33.9
25	0.620	0.210	33.8
50	0.704	0.246	34.9
60	0.700	0.238	34.0
65	0.700	0.230	32.8
80	0.684	0.246	35.9

Onset of cytokinesis, 75 minutes.

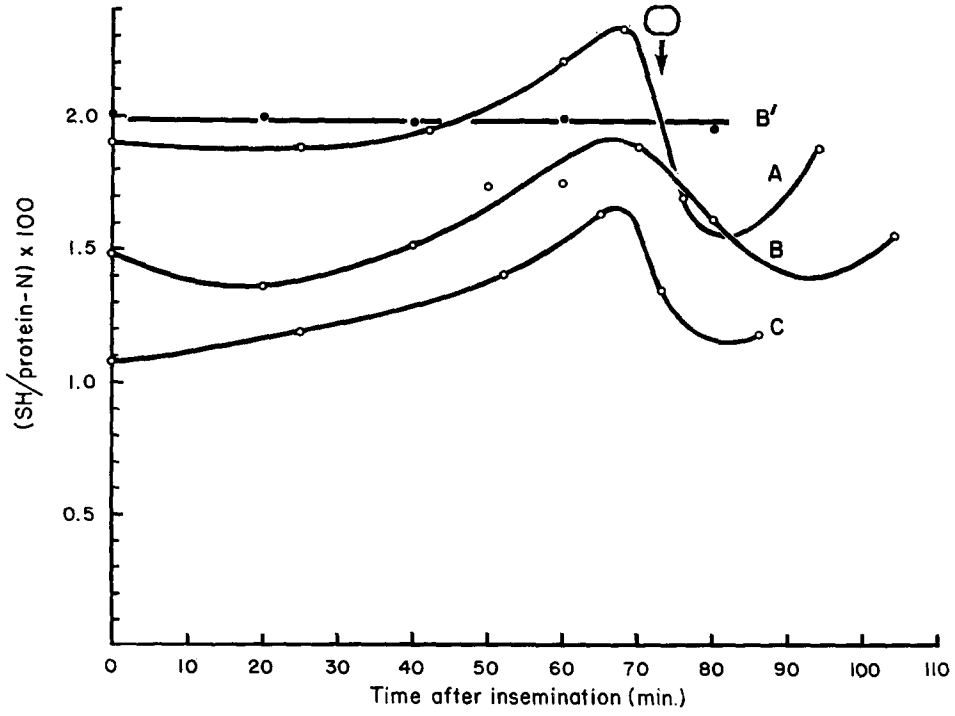

FIGURE 3

Change in (SH/protein-N) × 100 of KCl-soluble fraction of eggs during development. A, *Anthocidaris;* B, *Hemicentrotus;* C, *Pseudocentrotus;* B′, *Hemicentrotus*, SH + SS.

4. —*SH and —S—S— Groups in KCl-Soluble Egg Protein*

As was shown in Table II, the amount of N-KCl egg does not change during cell division. On the other hand, the SH/N-KCl egg increases gradually to a maximum at the meta-anaphase in all three sea urchin species. The advance of the cleavage furrow is accompanied by a decrease in SH/N. Thereafter it increases again toward the second division (Fig. 3). In other words, the mode of change in SH/N-KCl egg is seemingly similar to that of SH/N-TCA egg. Although the values of SH/N-KCl egg differ among the three sea urchin species, the range of the fluctuation is almost the same.

Now, the question arises, whether the change in SH/N-KCl egg is due to its freely reacting —SH groups or to sluggish and masked —SH groups, as classified by Hellerman *et al.* (4) and Barron (1). The procedure to test this point is divided into two steps: (*a*) measurement of —SH by using HCl-acetone and (*b*) oxidation of the freely reacting —SH to —S—S— by 0.01 M cystine before using HCl-acetone, followed by procedure (*a*). (*a*) − (*b*) gives the amount of freely reacting —SH, and (*b*) gives sluggish + masked —SH groups. From Fig. 4, it is clear that the fluctuation in —SH is due entirely to interchange between —SH and —S—S— within the freely reacting category. To reinforce this conclusion, BAL was used to reduce disulfide bonds on the HCl-acetone-denatured proteins of various stages of *Hemicentrotus* eggs. The results are shown in Fig. 3 (B′). After reduction by BAL, the values of —SH + —S—S—/N-KCl egg (B′ of Fig. 3) increase up to a constant level which is higher than the level of values before reduction (B of Fig. 3). This shows that interchange of —SH and —S—S— must be occurring among the readily available groups only.

5. —*SH Groups in KCl-Soluble Fraction of Cortex*

From the preceding section, the fluctuation in amounts of SH/N-KCl egg and of SH/N cortex (12) was found to be quite similar. Considering the probable importance of this phenomenon to cell division, the seat of the protein within the egg cortex which shows such a fluctuation was searched. To do this, the cortical hulls were fractionated into (*a*) a water-soluble fraction (by extracting at 0°C. for 3 hours); (*b*) a KCl-soluble fraction (by extracting the sediment of the former with 0.6 M KCl at 0°C. for 3 hours (KCl.cortex)); and (*c*) a KCl-insoluble residue. The water-soluble

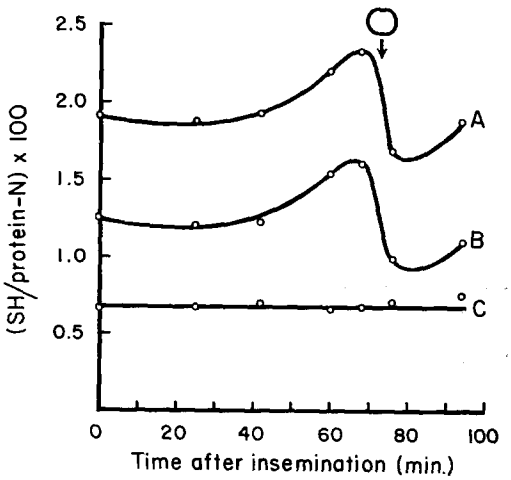

FIGURE 4

(SH/protein-N) × 100 of freely reacting and sluggish + masked—SH groups of KCl-soluble fraction of *Anthocidaris* eggs during development. A, total; B, freely reacting; C, sluggish + masked.

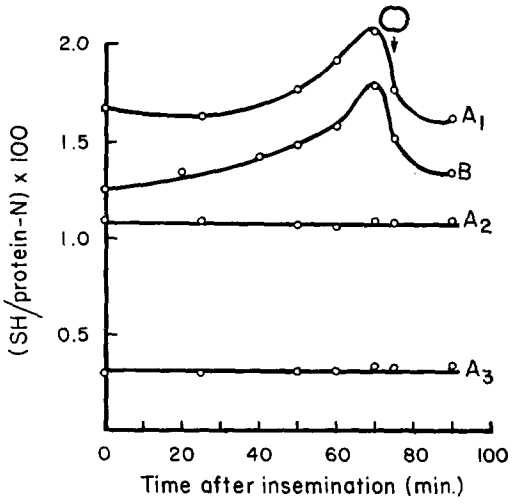

FIGURE 5

(SH/protein-N) × 100 of water-soluble, KCl soluble and KCl-insoluble fractions of egg cortex during development. A_1, KCl-soluble, *Hemicentrotus*; A_2, KCl-insoluble, *Hemicentrotus*; A_3, water-soluble, *Hemicentrotus*; B, KCl-soluble, *Pseudocentrotus*.

fraction (*a*) has a low SH/N value and neither (*a*) nor (*c*) fluctuates during the course of development. On the other hand, the SH/N-KCl cortex (*b*) shows a marked change, which is correlated with cell division (Fig. 5). The fluctuation is very similar to that of the SH/N-KCl egg. Thus the characteristic change in SH/N-cortex owes its origin to SH/N-KCl cortex.

DISCUSSION

The TCA-soluble and KCl-soluble proteins of the sea urchin egg exhibit many similarities as well as slight differences with respect to the pattern of the fluctuation of SH/N (compare Figs. 1 and 2 with 3). In spite of this, the TCA-soluble protein, if judged by its solubility and distribution, appears to constitute a part of the KCl-soluble fraction.

Since the morphological changes of cell division and the interchange between the —SH and —S —S— of freely reacting groups of the KCl-soluble fraction correlate well with each other, the fraction appears to have some share in the division activity. The values of SH/N of the total egg protein (SH/N egg) do not change during the course of development. This may imply either that some mirror image exchange of —SH is occurring between the KCl-soluble fraction and other fraction, or that the fluctuation of —SH of the KCl-soluble fraction is obscured by the presence of other proteins in an amount 9 times as great as that of the KCl-soluble fraction. The maximum value of SH/N is found at the stage in which the mitotic apparatus is growing, and its decrease corresponds to the disappearance of the mitotic apparatus. This fact is just opposite to the idea proposed by Mazia and Dan (9) that the formation and disintegration of the mitotic apparatus are directly connected with the formation and breakage of intermolecular —S—S— bridges, respectively. Although the behavior of —SH groups of the KCl-soluble fraction has turned out to be incompatible with the Mazia-Dan theory, its correlation to the cleavage activity in the reverse sense cannot be denied. As a matter of fact, Mazia found that the mitotic apparatus isolated in dithiodiglycol solution was easily dissolved in 0.5 M KCl (8). A possible role of the KCl-soluble fraction in cell division must therefore be sought in some other connection than the Mazia-Dan approach.

In plants, measurements of the —SH groups of the anther and microspores of *Lilium longiflorum* and *Trillium erectum* have been made by Stern (14–16), who found that the "soluble thiol" of the anther increased in concentration as the microspores approached division. On the other hand, the protein —SH underwent a gradual change in amount, while the protein disulfide showed a large increase, having a complicated correlation with mitosis. However, since the microspores undergo only nuclear division, the change in —SH values cannot be discussed along with the cytoplasmic division of sea urchin eggs.

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