STUDIES ON SULFHYDRYL GROUPS DURING CELL DIVISION OF SEA URCHIN EGG

II. Mass Isolation of the Egg Cortex and Change in Its —SH Groups during Cell Division

HIKOICHI SAKAI, Ph.D.

From the Department of Biology, Tokyo Metropolitan University, Tokyo, and the Misaki Marine Biological Station, Miura-shi, Japan

ABSTRACT

Masses of cortices of both unfertilized and fertilized sea urchin eggs can be isolated by crushing eggs in hypotonic $MaCl_2$ (0.1 M) solution. The amount of cortical material in terms of protein-N increases steadily after fertilization until the monaster stage and thereafter remains almost constant until well into the two-cell stage. The amount of bound —SH per protein-N of the egg cortex also increases after fertilization, reaches a maximum value at the amphiaster stage and thereafter decreases rapidly as the cleavage of the cell proceeds.

It is well known that the cortex of sea urchin eggs is a gelated structure. This is confirmed by centrifuging experiments, in which the endoplasmic components are easily stratified, whereas little effect is produced in the peripheral region.

Mitchison (6) and Hiramoto (5) have recently measured the thickness of the egg cortex. Hiramoto found that the thickness increases steadily after fertilization until the amphiaster stage; during cleavage, while the thickness further increases at the furrow region, it decreases at the polar regions.

Only histochemical information has been available so far on the chemical nature of the cortex. More reliable data could be obtained if egg cortices were isolated in a large amount.

Motomura (7) reported a method for the isolation of the cortex of sea urchin eggs which requires fixation with acid. Recently Allen obtained the cortical materials by stripping eggs which were attached to glass (1) and also by strong centrifugation (2). Allen's former method, however, is not suitable for mass analysis, and the strong centrifugal forces used in the latter method will disturb he arrangement of the cortical components so much that some of them may be lost in the process.

The present paper will describe a method for the mass isolation of the cortical material of sea urchin eggs and the changes incurred by its protein-N and bound —SH groups through a division cycle.

MATERIALS

The eggs of the sea urchins *Anthocidaris crassispina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus* were used; these forms are easily available at the Misaki Marine Biological Station.

METHODS AND RESULTS

1. *Isolation of the Egg Cortex*

The jelly layer of unfertilized eggs was removed with acidified sea water. After insemination, fertilization membranes were removed by urea, and the eggs were allowed to develop in the aerating apparatus previously described (11).

Eggs are first washed with cold 0.1 M $MgCl_2$ and crushed gently in the same solution by means of

This work was partly supported by a grant from the Rockefeller Foundation to Professor K. Dan.
Received for publication, January 28, 1960.

a glass homogenizer. To avoid excessive homogenization, the suspensions are frequently examined under the microscope. Once the cell membranes are punctured, the fluid endoplasm escapes and the residue which may be regarded as the cortex corresponding to Hiramoto's gelated layer is held intact by the presence of Mg ions. The cortical material is sedimented by centrifuging at 1000 R.P.M. for a short time and washed several times with cold 0.1 M $MgCl_2$.

Thus isolated, the outside surface of the cortical hulls of unfertilized egg appears smoother than that of fertilized egg, suggesting that the vitelline membrane may be preserving the smooth form of the former (Figs. 1 and 2). Moreover, it can be seen that fertilized cortical hulls are thicker than unfertilized hulls. With the present methods, it has so far been impossible to obtain dumb-bell-shaped hulls from dividing eggs.

Fig. 3 shows a sample prepared from unfertilized *Anthocidaris* egg. The hull has granules of uniform size, which stain with Janus Green B, and are arranged in a peripheral single layer characteristic of cortical granules. Fig. 4 shows intact unfertilized eggs as a reference for Figs. 1 and 2.

The cortical hulls of the eggs of the other sea urchins *Mespilia globulus*, *Temnotleurus toreumaticus*, and *Diadema setosum* can also be isolated by the same procedure.

2. *Protein-N of the Egg Cortex during Development*

A mass of isolated cortical hulls is treated with acetone to remove lipids. The nucleic acids are removed by 10 per cent TCA applied at 90°C. for 20 minutes. The micro-Kjeldahl-N of the residual material is regarded as protein-N.

Change in cortical protein-N is illustrated in Fig. 5 from which it can be seen that the protein increases on fertilization but remains constant throughout cleavage. The same is true for *Pseudocentrotus*, *Hemicentrotus*, and *Anthocidaris* eggs (Fig. 5).

3. *Bound —SH Groups in the Cortical Hulls during Development*

Bennett's procedure (3) was modified as follows. (*a*) Solvent of mercury orange. To avoid a steric hindrance of protein molecule to mercury orange by the use of toluene as Bennett experienced, acetone was chosen for its water miscible nature. (*b*) Reaction mixture for mercaptide formation. A *fresh* mixture of 0.2 M KH_2PO_4-NaOH buffer (pH 7.0), and an equal volume of acetone containing mercury orange was used. In this mixture the reaction completes itself within 1 hour in contrast to 1 to 7 days in toluene. (*c*) Direct measurement of protein-bound mercury orange. After protein is specifically stained by mercury orange, the dye is eluted in acetone containing a small amount of HCl.

In the above conditions, (*a*) the complete denaturation of proteins by HCl-acetone, (*b*) the specificity of mercury orange for protein-bound —SH groups, (*c*) the dissociation of mercaptide linkage by HCl, and (*d*) the reproducibility of data were tested for various proteins and found satisfactory.

The general procedure is as follows. Protein is

FIGURE 1

Isolated cortical hulls of unfertilized *Anthocidaris* eggs. (× *ca.* 200)

FIGURE 2

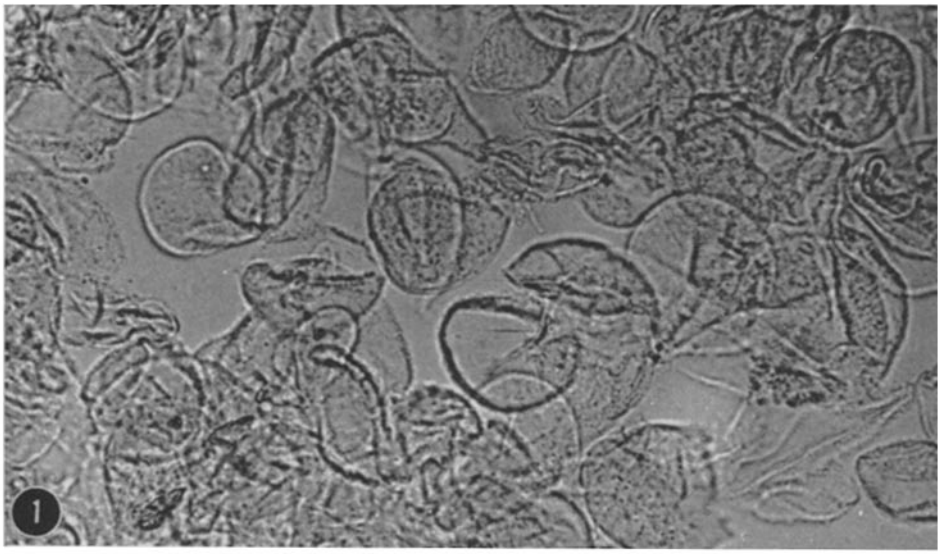
Isolated cortical hulls of fertilized *Anthocidaris* eggs (monaster stage). (× *ca.* 200)

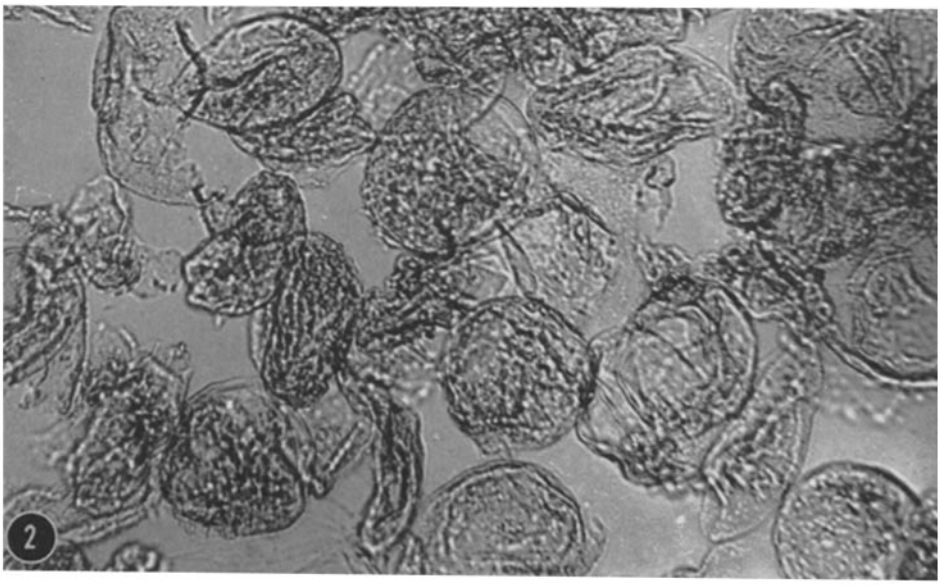
FIGURE 3

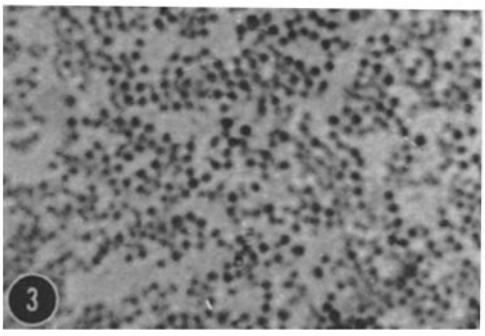
Granules in an isolated cortical hull stained by Janus Green B (surface view). (× *ca.* 2250)

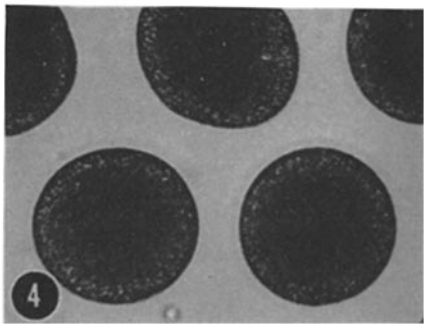
FIGURE 4

Unfertilized *Anthocidaris* eggs. (× *ca.* 200)









first denatured by treatment with *ca.* × 9 volume of HCl-acetone (0.06 M HCl in acetone), or guanidine hydrochloride, or urea. Then the denatured protein is added to the reaction mixture, ground into powder in a *glass-stoppered* centrifuge tube, and incubated at 25°C. for 1 hour with occasional stirring. After removing unbound mercury orange, the dye which is in a mercaptide combination with —SH is measured photometrically after its dissociation by HCl-acetone. Measurements were made on a Hitachi photoelectrometer, Type EPO-B, and the values of —SH groups were expressed as SH/protein-N (simplified as SH/N in the following pages).

A systematic change in the SH/N ratio is found in the course of development in both the *Pseudocentrotus* and *Hemicentrotus* cortices (Fig. 6). The SH/N value gradually increases after fertilization and reaches a maximum at the stage just before cleavage, after which it decreases rapidly as the cleavage furrow deepens. It is worth noting that by the time the cleavage furrow appears, the decrease in the amount of —SH has already begun both in the *Hemicentrotus* and *Pseudocentrotus* egg.

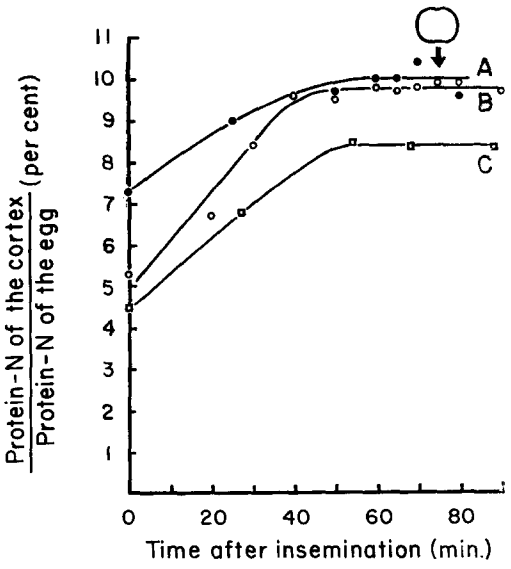


FIGURE 5

Percentage increase of protein-N of egg cortex during development. A, *Hemicentrotus*; B, *Pseudocentrotus*; C, *Anthocidaris*.

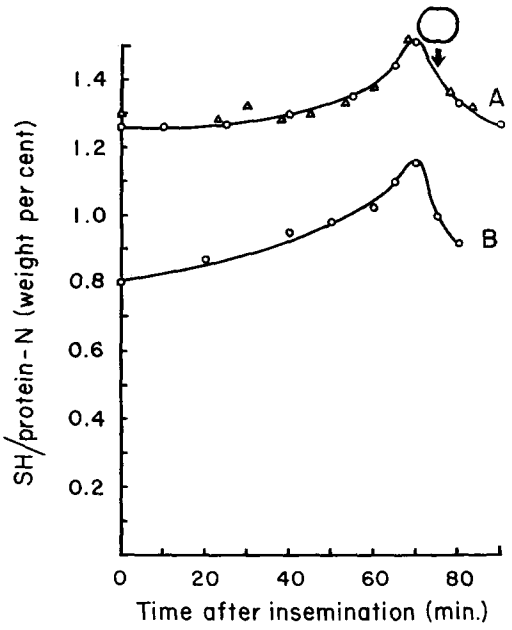


FIGURE 6

Change in the SH/protein-N ratio in egg cortices during development. A, *Hemicentrotus*, (○) denatured by HCl-acetone and (△) denatured by guanidine hydrochloride; B, *Pseudocentrotus* denatured by HCl-acetone.

DISCUSSION

Gelated cortical material, measured in terms of protein-N, increases after fertilization, as Hiramoto's physical measurements indicated (5). If it is assumed that proteins are evenly distributed throughout the cells, the thickness of the cortex calculated from the protein-N values agrees well with Hiramoto's figures.

After the monaster stage, the cortical protein-N remains almost unchanged even after cleavage. This suggests that the local differences in the thickness of the cortex at the furrow and polar regions so far reported (Dan and Dan (1940) and Hiramoto (1957)) may be caused by contraction and expansion within the cortex.

In view of the behavior of the —SH groups in the cortex, the possibility of chemical actions between the cortex and the endoplasm during cell division should be considered. What can be said at present is that the increase in —SH observed indicates a net gain of —SH and not an unmasking of hidden

—SH, because in the present study —SH value are expressed as —SH per protein-N in a thoroughly denatured state of the cortical proteins. Therefore, the possibilities left open are either reversible oxidation and reduction, —SH ⇌ —S —S — within the cortex, or exchange of —SH and —S —S — at the boundary between the cortex and the endoplasm which cannot be detected by available methods. Glutathione involvement has been ruled out by the data reported in the previous paper (11).

In contrast to the situation described in sea urchin eggs, Nickerson and Falcone (8–10) have reported that in baker's yeast, *Candida albicans*, a reduction of disulfide bridges takes place in the protein of the cell wall during budding. In budding, the daughter cell must project itself out of the mother cell, an operation that may necessitate a softening of the cell wall. On the contrary, in order to separate two blastomeres of the cleaving sea urchin egg, the maintenance of a certain degree of rigidity of the cortex may be required to prevent the rounding up and fusion of the incipient blastomeres.

The author wishes to express his thanks to Professor K. Dan for his valuable criticism and encouragement, and to Dr. Y. Endo for his kind suggestions. The author's thanks are also due to Professor H. Stanley Bennett of University of Washington for his generous gift of mercury orange, and to the Director and Staff of the Misaki Marine Biological Station for their kind help in many ways during the course of this work.

REFERENCES

1. ALLEN, R. D., *Exp. Cell Research*, 1955, **8**, 397.
2. ALLEN, R. D., *J. Cell. and Comp. Physiol.*, 1957, **49**, 379.
3. BENNETT, H. S., and WATTS, R. M., The cytochemical demonstration and measurement of sulfhydryl groups by azo-aryl mercaptide coupling, with special reference to mercury orange, *in* General Cytochemical Methods, **1**, (J. F. Danielli, editor), New York, Academic Press, Inc., 1958, 317.
4. DAN, K., and DAN, J. C., *Biol. Bull.*, 1940, **78**, 486.
5. HIRAMOTO, Y., *Embryologia*, 1957, **3**, 361.
6. MITCHISON, J. M., *Quart. J. micr. Sc.*, 1956, **97**, 109.
7. MOTOMURA, I., *Sc. rep. Tôhoku Univ. Biol.*, 1954, **20**, 318.
8. NICKERSON, W. J., and FALCONE, G., *Science*, 1956, **124**, 318.
9. NICKERSON, W. J., and FALCONE, G., *Science*, 1956, **124**, 722.
10. NICKERSON, W. J., and FALCONE, G., Function of protein disulfide reductase in cellular division of yeasts, *in* Sulfur in Proteins, (R. Benesch, R. E. Benesch, P. D. Boyer, I. M. Klotz, W. R. Middlebrook, A. G. Szent-Györgyi, and D. R. Schwarz, editors), New York and London, Academic Press, Inc., 1959, 409.
11. SAKAI, H., and DAN, K., *Exp. Cell Research*, 1959, **16**, 24.