A Cytochemical Study of the Sulfhydryl Groups of Sea Urchin Eggs during the First Cleavage

BY NAOKO KAWAMURA* AND KATSUMA DAN, PH.D.

(From the Department of Biology, Tokyo Metropolitan University, Tokyo)

PLATES 295 TO 297

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ABSTRACT

In the eggs of four species of echinoderms, *Mespilia globulus*, *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus* and *Clypeaster japonicus*, changes in the distribution of protein-bound SH groups from fertilization to the 2 cell stage have been studied cytochemically by use of a mercaptide-forming azo dye.

In the eggs of these species, the color intensity in the cytoplasm increased upon fertilization. The astral centers and spindle during mitosis were stained deeply. When the aster formation was suppressed by ether, hyaline spots appeared in the egg cytoplasm instead of well formed astral centers and these spots were stained by the SH-specific dye. Upon recovery of such eggs in pure sea water, and when cleavage ensued, such spots disappeared and two new astral centers were reorganized.

The SH-protein occurring in the centrosphere is considered to be the precursor material for the asters and spindle, and this material is apparently derived from the cytoplasm.

In the study of the mechanism of cell division, a knowledge of the structure and composition of asters and spindle is of the utmost importance. Monné (25), Hughes and Swann (11), Inoué and Dan (15) and Inoué (13, 14) studied the structure of the mitotic apparatus with the polarization microscope. Inoué and Dan have suggested that the rays of mitotic apparatus are well defined entities, composed of a gel of protein nature. Recently Mazia and Dan (22) reported on the chemical composition of the isolated mitotic apparatus. They proposed the hypothesis that the fibrous protein of the mitotic apparatus consists of unit protein molecules containing sulfhydryl (SH) groups which are oxidized during spindle formation, forming disulfide (S—S) bridges between adjacent protein molecules.

In the present investigation, the changes in distribution of protein-bound SH groups in sea urchin eggs from fertilization to the 2 cell stage were studied cytochemically under the hypothesis stated above.

* Present address: Department of Zoology and Entomology, University of Tennessee, Knoxville.

Materials and Methods

Four species of echinoderms available at the Misaki Marine Biological Station were studied comparatively. These include the sea urchins, *Mespilia globulus*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*, and the heart urchin, *Clypeaster japonicus*.

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, monaster, streak, mitosis, and the 2 cell stage. Ether–sea water was used as an inhibitor of cell division.

As fixatives, 2, 5, and 8 per cent trichloracetic acid, 10 per cent formalin, 30 per cent cold ethanol and cold absolute acetone were used. Fixed materials were embedded in paraffin on the following day and sectioned. Protein-bound SH groups were detected by the staining methods of Barrnett and Seligman (1, 2), Bennett (3), Chèvremont and Frederic (6), Tahmisian and Brues (30), and Giroud and Bulliard (10).

1. *Determination of Specificity for SH Groups of Various Staining Methods.*—

In order to compare the natures of the different stains, the following five dye stuffs were tested under blocked and unblocked condition of SH groups. For

the purpose of blocking, M/200 *p*-chloromercuribenzoic acid (PCMB), and 15 per cent H_2O_2 in the presence of catalytic amounts of iron ion were employed, and as test objects, unfertilized and cleaving eggs of *Pseudocentrotus depressus* as well as striated muscle of the frog were used. Both blocked and unblocked sections were stained side by side. The mere fact that sections cease to be stained by an SH dye after treatment with a blocking agent does not necessarily mean that the dye has the highest specificity for SH, since the accessibility of the dye to the SH radicals seems to depend to some extent upon the molecular configuration. Conversely, when a dye is still taken up after blocking, there is a possibility that the blocking may not have been efficient. It is also possible that the dye may be combining with other than SH radicals. Since it is difficult to make a distinction between the last two alternatives, a dye, that is completely ineffective in staining after SH blocking, was sought.

A. Bennett's reagent, 1-(4-chloromercuriphenylazo)-naphthol-2:[1] The specificity of this reagent for SH groups was examined in butanol solution (Bennett, (3)) and ethanol solution (Mescon and Fresch, (23)). Whereas striated muscle was stained equally well by the reagent in both types of alcohol, the protein-bound SH groups of the sea urchin eggs were stained much more heavily in ethanol than in butanol. The striated muscle and the sea urchin eggs blocked by PCMB or H_2O_2 were not stained at all in either alcohol.

B. Barrnett and Seligman's method: The muscle and sea urchin eggs stained by this method were deep reddish-purple. Some reduction in the color of PCMB- and H_2O_2-treated section was noted, although not nearly to the degree observed when Bennett's reagent was used. The color after blocking was reddish.

C. Tahmisian and Brues' cobalt and BAL method: In the control sections, the color of the stained specimens was brown, while in the sections treated by PCMB or H_2O_2, it became fainter, but, as in Barrnett and Seligman's method, it was not completely eliminated.

D. Chèvremont and Frederic's method: By this method, although the materials were stained a deep prussian blue, the color was scarcely affected after treatment with PCMB. In sections treated with H_2O_2, the color of the materials became only slightly fainter.

E. Nitroprusside: When subjected to Giroud and Bulliard's nitroprusside method, the sectioned striated muscle was stained a pale pinkish color, but the sections of sea urchin eggs remained entirely colorless.

From the results obtained here, it is our opinion that the reagents of Barrnett and Seligman, Tahmisian and Brues, and Chèvremont and Frederic may combine with some radicals of protein other than SH groups, because there remained a certain residual amount of coloration even after the SH groups were blocked by PCMB or H_2O_2. Nitroprusside is also unsuitable for this cytochemical purpose, because this method is functionally insensitive to the minute amounts of SH groups present in the sectioned eggs. Since Bennett's reagent fulfills the condition hoped for better than other reagents, this method was mainly adopted.

2. Fixatives for Staining by Bennett's Reagent.—Danielli (9) and others have recommended acetone, formalin, alcohol, and trichloroacetic acid as fixatives for SH groups. In the present experiment, sections of sea urchin eggs fixed in these solutions were stained by Bennett's reagent in both butanol and ethanol. The best result was obtained when the eggs were fixed in 5 per cent trichloroacetic acid followed by paraffin embedding on the following day and when sections were stained by the dye dissolved in 80 per cent ethanol.

The staining procedure was as follows:—

1. The eggs were fixed in 5 per cent trichloroacetic acid for 12 hours.
2. Washed in several changes of distilled water for 30 minutes.
3. Dehydrated by serial transfers through graded ethanol solutions, and embedded in paraffin through xylol.
4. Sectioned at 15 μ and mounted on slides without albumen.
5. Paraffin removed by xylol, and sections hydrated to 80 per cent ethanol.
6. Stained for 3 hours by Bennett's reagent saturated in 80 per cent ethanol.
7. Washed free from excess reagent in several changes of 80 per cent ethanol for 24 hours.
8. Dehydrated, and mounted in balsam.

By this procedure the region containing SH groups is stained faintly orange, a color which is rather difficult to discern. Consequently, a filter that is pervious to blue and a part of the red light was prepared by staining a photographic film to a suitable intensity with toluidine blue. Since the region stained by the reagent transmits red and yellow light, it stands out more clearly as a red area on a blue background.

[1] The reagent was generously supplied by Prof. H. S. Bennett of the University of Washington, Medical School, to whom the authors express their deep gratitude.

RESULTS

1. Observations in Normal Eggs.—

The changes in protein-bound SH group distribution during development were almost the same among the four species of echinoderms used in these experiments. Since the time required from fertilization to the first cleavage of these echinoderm eggs varied from 30 to about 100 minutes between the summer and winter species, this discrepancy in developmental rates was taken into consideration in obtaining the stages listed below.

Unfertilized egg: The cytoplasm and nucleus in unfertilized eggs showed almost no coloration in the untreated eggs (Figs. 1 and 13) and none in the experimental groups treated with PCMB or H_2O_2.

Immediately after fertilization: The cytoplasm of the egg showed an increased stainability upon fertilization, whereas both the egg and sperm nuclei remained unstained. Likewise, the sperm aster developing in the egg cytoplasm was not stained (Figs. 2 and 14).

Monaster stage: The fusion nucleus began to take on the stain during this stage (Figs. 15 and 16).

Streak stage: In streak stage eggs, the nucleus and so called "streak" showed deep color (Figs. 3 and 17).

Mitosis stage: At the late streak stage, the color of the nucleus became fainter concurrently with the increase in nuclear volume (Fig. 18). At the poles of the now ovoid nucleus, there were deeply stained astral centers (Fig. 19). Thereafter, a stained portion of the astral center grew larger and the color became more intense. Just before the disappearance of the nuclear membrane, fibrous structures connecting the astral centers appeared. After the nuclear membrane disappeared, the astral centers and spindle took up more stain as they developed, until the color reached the highest intensity at late prophase and metaphase. The chromosomes were also deeply stained (Figs. 4 to 8, 20 and 21).

At anaphase, the color of the astral centers became fainter with an increase in their cross-sectional area. As the chromosomes separated, the interzonal region began to fade, the loss of color beginning at the midpoint of the spindle (Figs. 9, 10, and 22).

At telophase the spindle was almost invisible, except for the portions near the daughter chromosomes, and the astral centers were only faintly stained. The reconstituted daughter nuclei were not stained, while the surrounding cytoplasm retained a faint color (Figs. 11 and 26).

When one observes the granular, opaque eggs of *Pseudocentrotus* and *Hemicentrotus* in the living state, a portion of the spindle and the astral centers (centrosphere or astrosphere) appears transparent. This whole complex has the shape of an hour glass at metaphase. It then gradually flattens in the direction of the spindle axis, resulting in a butterfly shape at early telophase and an H shape at late telophase. Such a form change of the centrospheres has been reported in sectioned marine eggs by Yatsu (32) and Just (16).

When these eggs were stained by SH reagents, the astral centers and spindle showed the same changes in shape as the centrospheres of the living eggs (Figs. 21 to 25). By the methods of Barrnett and Seligman, of Tahmisian and Brues, and of Chèvremont and Frederic, the astral centers and spindle were also stained deeply. When the eggs were stained after treatment with PCMB or H_2O_2, the astral centers and spindle remained entirely unstained, although the cytoplasm still showed some color.

Two cell stage: The daughter nuclei of the 2 cell stage again lost their stainability as in the earlier stages. The blastomere cytoplasm was stained only very faintly. In *Hemicentrotus*, however, the cytoplasm near the nucleus retained a trace of the color (Figs. 12 and 27).

2. Observations on Etherized Eggs.—

Wilson (31) has reported detailed observations on the suppression of aster formation in etherized eggs. Recently Dan (7) and Swann (29) have used ether for the same purpose in their experiments.

In the present study, the eggs of *Hemicentrotus pulcherrimus* were used. The eggs, treated with sea water containing ether in a concentration greater than 1 per cent, could perform neither cytokinesis nor karyokinesis, because of the suppression of both the asters and the spindle. On the other hand, eggs subjected to 0.6 per cent ether–sea water (ESW) underwent karyokinesis, but this was not accompanied by cytokinesis because of the failure of aster formation. Binucleate eggs resulted. Sea water containing 0.6 per cent ether, however, has no effect whatsoever on fertilization, syngamy or monaster formation.

Eggs were fertilized in ESW of this concentration, and retained in it throughout one experiment. After the disappearance of the nuclear membrane, these eggs did not show normally developing centrospheres. Instead, besides the hyaline nuclear substance, which was not necessarily localized in the central part of egg, there appeared ten or more hyaline spots in the egg cytoplasm. These hyaline spots could be observed most distinctly at the time when the control eggs were in first cleavage. As karyokinesis of the eggs in ESW proceeded, these spots gradually faded, and in the binucleate interphase eggs, they disappeared. When such eggs went into the second karyokinesis, the hyaline sports reappeared in the egg cytoplasm. If the eggs with hyaline spots were removed to normal sea water quickly enough, the hyaline spots disappeared and asters and spindle reformed gradually, followed by an abnormal cleavage.

The changes in distribution of SH groups coincided well with the distribution and behavior of hyaline spots in the living etherized eggs. Sections of these eggs revealed a larger area of nuclear substance, along with deeply stained spots in the egg cytoplasm (Figs. 28 to 36). When subjected to the nitroprusside test, the hyaline spots of living eggs showed an intense color reaction.

When the etherized eggs were stained with Heidenhain's hematoxylin or brom-phenol blue (Mazia, Brewer, and Alfert, (21)), only a miniature aster and spindle could be seen in each, but no structure corresponding to the hyaline spots could be observed.

DISCUSSION

Quantitative estimations of soluble SH groups in relation to cell division have been reported by Rapkine (26), Infantellina and LaGrutta (12), and Bolognari (4), with glutathione primarily in mind. Rapkine stated that the amount of SH groups extracted with 25 per cent trichloroacetic acid was minimal at the monaster stage, then increased to a peak just before cytokinesis. Cytochemical study of SH groups has been performed only by Brachet (5), who used amphibian eggs fixed by trichloroacetic acid. By the nitroprusside method he observed abundant SH groups in the nuclear sap, aster, and spindle.

The first fact revealed by the present study is that the eggs of four species of echinoderms show an increased stainability of protein-bound SH groups upon fertilization. Although fertilized eggs shrank perceptibly more than unfertilized eggs during the process of paraffin embedding, (with the volume of the fertilized eggs on occasion becoming reduced to one-half that of the unfertilized eggs), it does not seem that the increase in color intensity is attributable to the shrinkage of the eggs alone. The second fact is that the astral centers and spindle are deeply stained by SH reagents. In sections of the eggs it can be seen that it is the fibrous structure of the asters and spindle that is stained by SH reagents, an observation verified by electron microscopy (unpublished). The stained pattern of the asters and spindle coincides with the configuration reported by Inoué (14) on the basis of birefringence microscopy.

The birefringence studies of the spindle and asters made by Inoué and Dan (15), Swann (28) and Inoué (13, 14) all point to the likelihood that the asters and spindle are constructed from well oriented micelles. Mazia and Dan (22) suggested that adjacent protein molecules in asters and spindle might be united in linear sequence by intermolecular S—S bridges in a fibrous form. In the present observation it has been shown that the protein component of the asters and spindle abounds in SH groups. However, in order to ascertain whether or not these SH proteins are combined by S—S bridges, a further study must be carried out.

Lorch (19) established the following two points by her microdissection experiments on sea urchin blastomere. (*a*) As long as the centrospheres were left intact, asters could be regenerated in the succeeding division cycle in spite of the absence of the nucleus. (*b*) On the other hand, if the nucleus alone was left, a considerable lag period intervened before centrospheres were regenerated and only after this, asters could be formed. In the present experiment it has been shown that the localization of SH groups in the eggs at mitosis coincides with the centrosphere, and if the formation of aster is suppressed by ether, several hyaline spots abundant in SH groups appear in a random fashion throughout the egg cytoplasm instead of a centrosphere. Such hyaline spots appear at the time corresponding to the stage of high mitotic activity and disappear at the time of the resting stage in control eggs. It seems that the SH protein occurring in the centrosphere or hyaline spots is the material of the aster and the spindle.

After treatment by PCMB or H_2O_2, if eggs

were stained by the methods of Barrnett and Seligman, Tahmisian and Brues, and Chèvremont and Frederic, the centrosphere remained colorless, while the cytoplasm retained its color. Although the coloring of cytoplasm does not necessarily show radicals other than SH group, the colorless centrosphere shown by each staining reaction indicates that the same type of SH protein, which can combine with the mercaptide-forming agent, can concentrate in the region of the centrosphere. We thus consider that one of the functions of the centrosome is to collect materials preparatory to the formation of asters and spindle, as previously suggested by Mazia (20) and more recently by Dan (8). The ether may act as a blocking agent for such a function of the centrosome.

As for the nucleus, it is rather unexpected to find it unstainable in interphase. In prophase it gradually begins to pick up the stain, and in very late prophase, but definitely before the dissolution of the nuclear membrane, the centers of the small paired asters and a spindle-shaped connecting structure between the asters begin to stain rather deeply. At metaphase the stainability reaches its maximum, and the now fully developed centers and spindle and chromosomes are intensely colored. In other words, the intercleavage period can be divided roughly into two phases, namely, the phase of general low stainability and that of localized stainability. The second phase is initiated by the acquisition of stainability by the nucleus.

It does not necessarily follow, however, from these observations that the mitotic apparatus owes its material entirely to the nucleus. In the present study, a precursor spindle has been shown to exist bridging the space between the two astral centers already before the dissolution of the nuclear membrane. As a matter of fact, Meves (24), Lams (18), Shimakura (27), and Kawamura, K. (17) have made similar findings in an amphibian, a mollusc and a grasshopper. It may well be that the component materials of asters and spindle come from the cytoplasm in echinoderm eggs as well.

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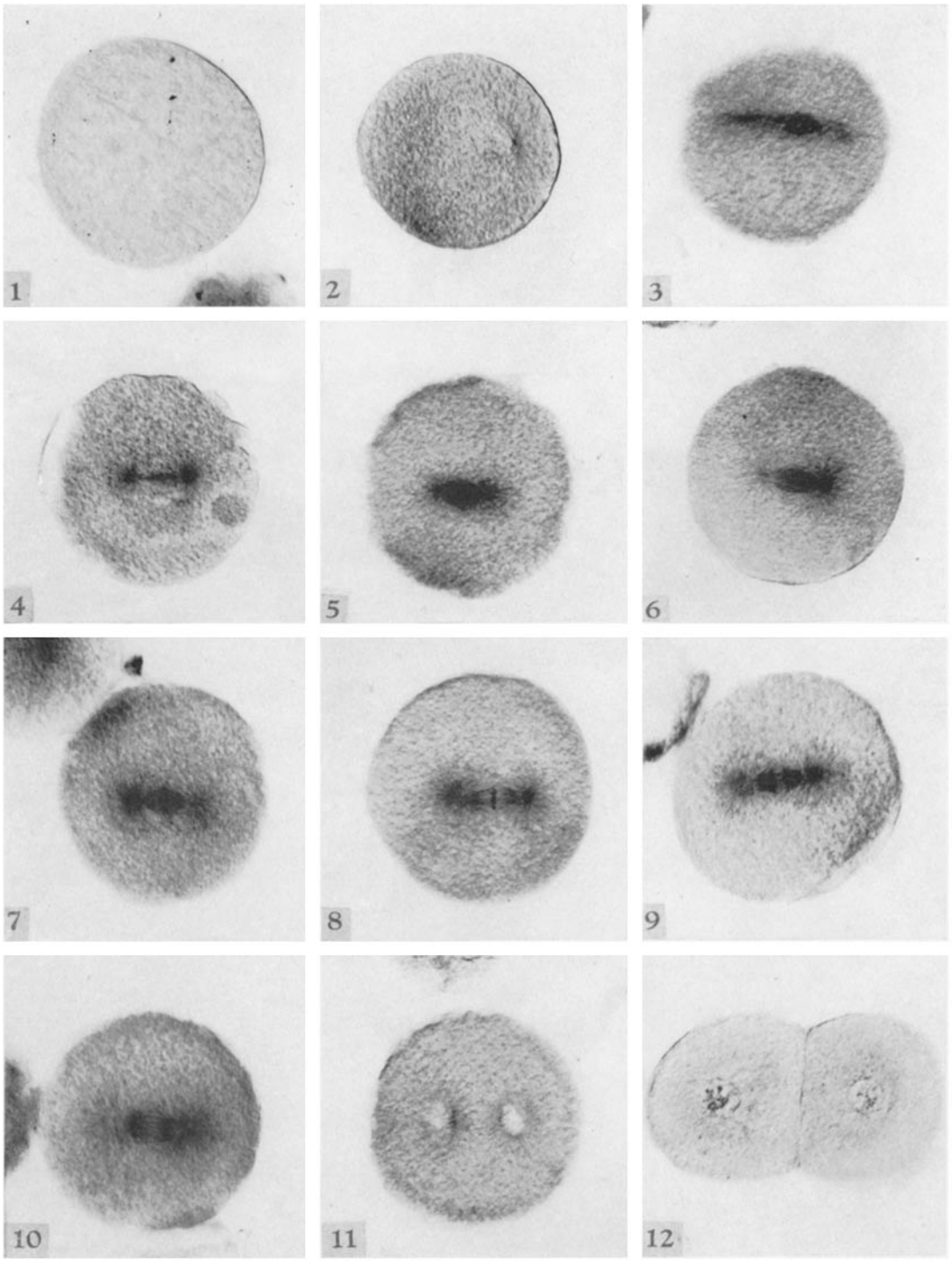
Explanation of Plates

Plate 295

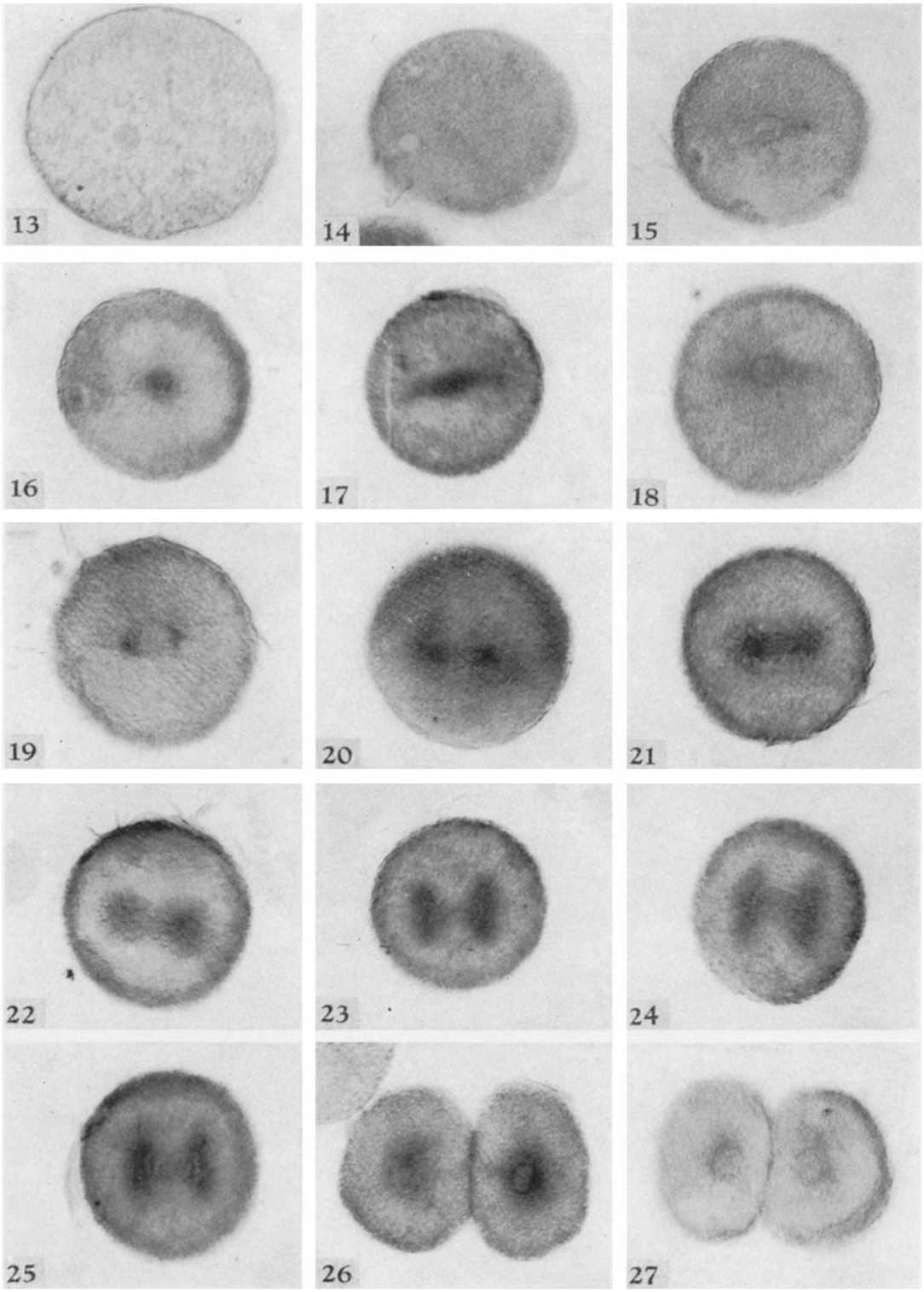
Figs. 1 to 12. The eggs of *Clypeaster japonicus*. Fixed in 5 per cent trichloroacetic acid and stained with Bennett's reagent. Fig. 1, unfertilized egg. Fig. 2, 3 minutes after fertilization. Fig. 3, streak stage. Figs. 4 and 5, prophase. Figs. 6 and 7, prometaphase. Fig. 8, metaphase. Figs. 9 and 10, anaphase. Fig. 11, telophase. Fig. 12, 2 cell stage. × 380.



(Kawamura and Dan: Sulfhydryl groups of sea urchin eggs)

PLATE 296

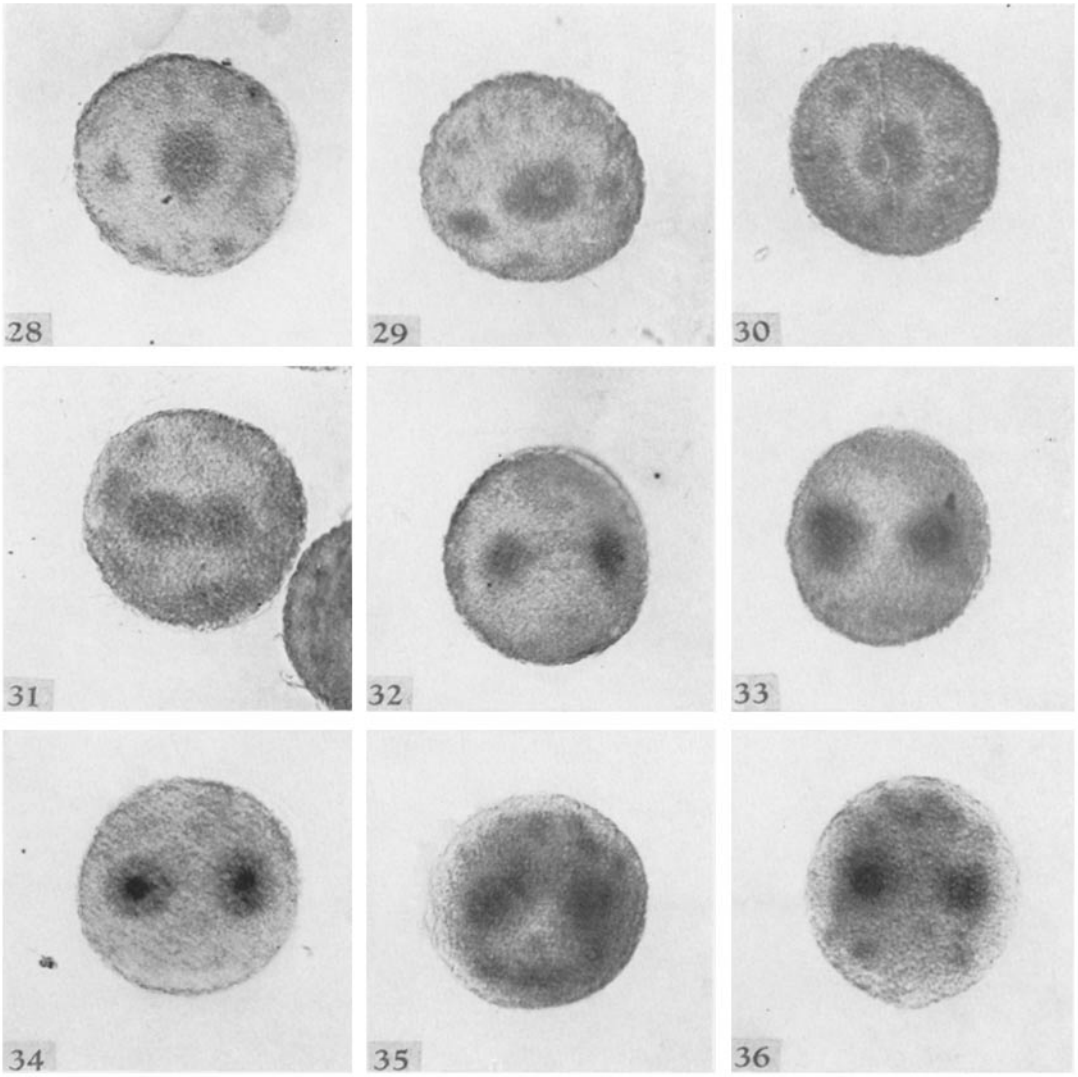
FIGS. 13 to 27. The eggs of *Hemicentrotus pulcherrimus*. Fixed in 5 per cent trichloroacetic acid and stained with Bennett's reagent. Fig. 13, unfertilized egg. Fig. 14, 5 minutes after fertilization. Fig. 15, syngamy stage. Fig. 16, monaster stage. Fig. 17, streak stage. Figs. 18 and 19, late streak stage or early prophase. Fig. 20, prophase. Fig. 21, metaphase. Fig. 22, anaphase. Figs. 23 to 25, telophase. Fig. 26, early 2 cell stage. Fig. 27, 2 cell stage. × 390



(Kawamura and Dan: Sulfhydryl groups of sea urchin eggs)

PLATE 297

FIGS. 28 to 36. The etherized eggs of *Hemicentrotus pulcherrimus*. Fixed in 5 per cent trichloroacetic acid and stained with Bennett's reagent. Figs. 28 to 30, eggs in the first karyokinesis. Hyaline spots appeared in the egg cytoplasm. Figs. 31 to 33, nucleus separated to both poles. Fig. 34, resting stage (binucleate egg). Figs. 35 and 36, eggs in the second karyokinesis. Hyaline spots reappeared. × 390.



(Kawamura and Dan: Sulfhydryl groups of sea urchin eggs)