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# GLUTATHIONE REDUCTASE IN THE SEA URCHIN EGG

## II. CLEAVAGE-ASSOCIATING FLUCTUATION OF THE ACTIVITY AND ITS POSSIBLE REGULATORY MECHANISM

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SUMMARY

The activity of glutathione reductase (NADPH:GSSG oxidoreductase, EC 1.6.4.2) in the sea urchin egg fluctuated periodically during the cleavage cycle. It decreased significantly during cell division and increased toward the following division cycle. Glutathione reductase activity in the water-soluble fraction was activated by EDTA- or ethyleneglycol-bis(aminoethylether)-*N,N'*-tetraacetic acid-treatment and the treatment diminished the fluctuation detected during the division cycle. By chromatography of the water-soluble fraction on Sephadex G-200, the enzymic activity was separated into the flow-through fraction and a retarded fraction. Only a complex form of enzyme in the G-200 flow-through fraction was activated by EDTA or ethyleneglycol-bis(aminoethylether)-*N,N'*-tetraacetic acid. The activity in the retarded fraction corresponded to that of the free enzyme. After activation through EDTA-treatment, the complex form of enzyme was converted to the free enzyme. The elution pattern of the complex form (in the G-200 flow-through fraction) on Sepharose 4B was broad (mol.wt 700 000–3 000 000), but sucrose density gradient centrifugation showed that the sedimentation pattern of the complex form was similar to that of the free form of enzyme. It was suggested that a lipoprotein might be the inhibitor.

## INTRODUCTION

It has been previously shown that the amounts of GSH and GSSG do not change during the cleavage cycle of the sea urchin egg [1,2]. This fact has led to the conclusion that the tripeptide would not have a direct role in cytokinesis. However, this could not deny a possible role of glutathione in cell division. The possibility remains that the equilibrium between GSH and GSSG is stable in the embryonic cell, so that simple analysis of the amount by a chemical method fails to detect the rapid change because of a quick recovery of the equilibrium through the GSSG–glutathione reductase system. Considering a high concentration of GSH within the cell, and func-

Abbreviation: EGTA, ethyleneglycol-bis(aminoethylether)-*N,N'*-tetraacetic acid.

tional roles of GSH or –SH groups in sugar metabolism and protein synthesis, investigations have been focussed on the enzyme system in the cell.

Fredborg and Lindahl [3] have shown that glutathione reductase activity in Ehrlich ascites tumor cells decreased in mitosis. Our results demonstrate a cyclic change in the activity during the cleavage cycle and present a possible mechanism for the regulation of the activity.

## MATERIALS AND METHODS

### *Biological materials*

The sea urchins, *Pseudocentrotus depressus*, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* were used. Gametes were obtained by 0.5 M KCl-induced spawning or injection of 0.1 M acetylcholine into the body cavity of the animals [4].

### *Assay of glutathione reductase activity*

Assay of the activity was carried out as described previously [5]. Protein assay was carried out according to the method of Lowry et al. [6] with bovine serum albumin as a standard.

### *Assay of glutathione reductase activity during division cycles*

Eggs were washed three times with filtered sea water before use, and fertilized. Culture was performed with constant stirring at room temperature. Only batches of embryos whose fertilization rates were nearly 100% and cell divisions were highly synchronous were used for this series of experiments. At each stage of the cell cycle, 10 ml of egg suspension were taken out and the eggs were collected by low speed centrifugation. After washing with cold water, packed eggs were homogenized in about 10 vol. of chilled water with a Teflon homogenizer employing seven strokes. The homogenate was centrifuged at 15 000 $\times$ $g$ for 15 min and the supernatant (water-soluble fraction) was dialysed against two changes of 50 vol. of 5 mM phosphate buffer (pH 7.6). The enzymic activity was determined in 100 $\mu$l of the dialysed water-soluble fraction.

### *Preparation of a postmicrosomal supernatant and microsomal fractions*

Fertilized eggs were collected by low speed centrifugation. After being washed with cold 0.74 M mannitol containing 5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.6), eggs were homogenized in about 10 vol. of the same solution. The homogenate was centrifuged at 12 000 $\times$ $g$ for 30 min and the precipitate discarded. The supernatant was re-centrifuged at 100 000 $\times$ $g$ for 60 min. The precipitate was washed with the same solution and designated as a "microsomal fraction". The supernatant was called "postmicrosomal supernatant". For the assay of the enzymic activity, both fractions were dialysed against 3 changes of 20 vol. of 5 mM potassium phosphate buffer (pH 7.6).

## RESULTS

### *Fluctuation of glutathione reductase activity during division cycles*

Glutathione reductase activity was determined with the dialysed water-soluble fraction of the sea urchin egg through two division cycles. Fig. 1 shows an example in

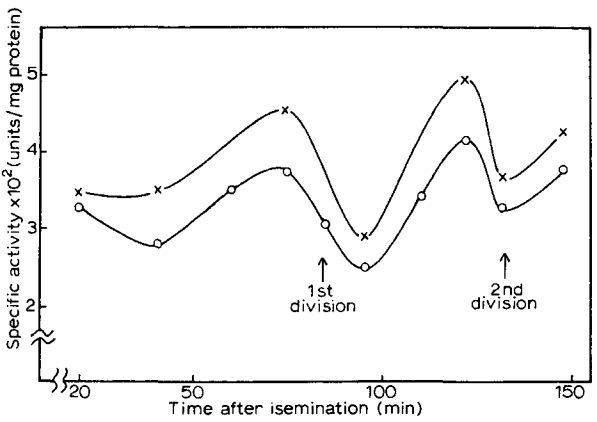


Fig. 1. Fluctuation of glutathione reductase activity during division cycles of the sea urchin egg (*Pseudocentrotus*). ○—○, assayed in 0.1 M potassium phosphate buffer (pH 7.2) without EDTA; ×—×, assayed in 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA.

*Pseudocentrotus*. The activity increased before the first cleavage and decreased significantly during cell division. After the cells divided, it increased about 2-fold toward the second division and decreased during the second cleavage. A rather similar fluctuation of the activity during cleavage cycles was also observed in *Hemicentrotus* and *Anthocidaris*.

This fluctuation of glutathione reductase activity was first found using an assay system containing no EDTA. Later, it was also demonstrated using the assay system containing 1 mM EDTA, though the apparent activity was increased by 20% as a whole. The same fluctuation was also observed in the dialysed postmicrosomal supernatant. The fluctuation was not caused artificially by dialysis, for a rather similar fluctuation of the activity was observed with the water-soluble fraction omitting dialysis. Since almost all of glutathione reductase activity was found in every supernatant obtained from embryos at various stages of the cell cycle, the fluctuation of the activity during the cell cycle was not considered as being due to insufficient extraction. Therefore, it was concluded that the glutathione reductase activity actually fluctuates, being closely associated with cell division.

A question arises as to how glutathione reductase activity can be regulated during a division cycle of the sea urchin egg. Does the quantity of the enzyme fluctuate, or does an activator or inhibitor contribute to the regulation of the activity?

Fredborg and Lindahl [3] suggested in Ehrlich ascites tumor cell that an activator or inhibitor may control glutathione reductase activity. This suggestion was brought out from their results that the activity of the enzyme was inversely proportional to a power of the frequency of cells in mitosis. Although we examined the activity in a mixture of two samples of both high and low activity, the combined activity was merely additive. The result is different from that of Fredborg and Lindahl. However, we cannot conclude from this result alone that the quantity of glutathione reductase in the sea urchin egg fluctuates.

It was found that the activity of glutathione reductase in the dialysed water-soluble fraction increased significantly after incubating at 25 °C for a few hours in 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2). The activity in

each sample obtained at each stage of the cell cycle increased to an activated level identical to each other, when incubated for more than 4 h as shown in Fig. 2. A similar activation was observed by ethyleneglycol-bis(aminoethylether)-*N*,*N*'-tetra-acetic acid (EGTA) in place of EDTA. Although incubation in 0.2 M phosphate buffer alone allowed an activation of the crude enzyme fraction to some extent, addition of EDTA or EGTA greatly enhanced the activity.

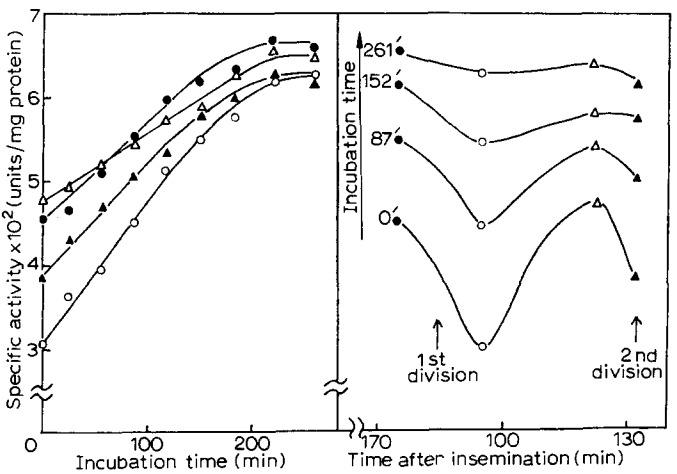


Fig. 2. Time course of increase in glutathione reductase activity in each sample incubated in 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C. The water-soluble fractions were obtained from eggs at 75 (●), 95 (○), 122 (△) and 132 min (▲) after insemination.

These results showed that the quantity of glutathione reductase does not fluctuate during cell division and that an inhibitor may be responsible for the regulation of the enzyme activity.

*Separation on Sephadex G-200 of two forms of glutathuone reductase*

A chromatogram of the water-soluble fraction on Sephadex G-200 is shown in Fig. 3. Glutathione reductase activity appeared not only in a fraction as anticipated from its molecular weight (tube No. 70-85), but also in the flow-through fraction (tube No. 46-56). Only the enzyme involved in the flow-through fraction was activated by EDTA or EGTA. Fig. 4A shows the time course of the activation by EGTA. The activity in the G-200 flow-through fraction was increased about 2-fold after incubation for 5 h, but the enzyme fraction retarded through G-200 and anticipated as being a free form of enzyme was hardly activated. The purified glutathione reductase was, of course, not activated by EGTA.

Subsequently, the G-200 flow-through fraction treated with EGTA was applied again on the Sephadex G-200 column. Fig. 4B shows the elution profile. Part of the activity remained in a fraction of the void volume, but most of the activity (more than 70% of the total) shifted from the G-200 flow-through fraction to the free form of enzyme. These results indicate that the enzyme in the G-200 flow-through fraction

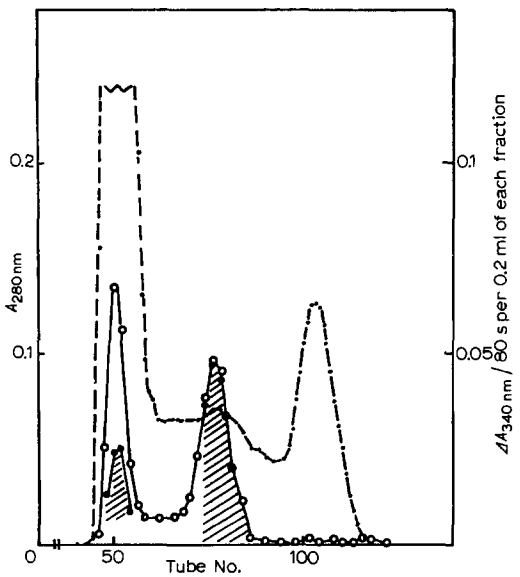


Fig. 3. Chromatogram of the water-soluble fraction on Sephadex G-200. A Sephadex G-25 flow-through fraction (6 ml, 50 mg protein) of the water-soluble fractoin (*Anthocidaris*) was applied to a Sephadex G-200 column (3 cm × 68 cm). Each fraction of 4.3 ml was analysed for protein and enzymic activity. ·---·, absorbance at 280 nm; ●—●, glutathione reductase activity before incubation with EDTA; ○—○, glutathione reductase activity after incubation in 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C for 7–8 h.

is a complex form with some higher molecular inhibitors and that the complex form can be dissociated to the free form of enzyme by EDTA or EGTA.

The chromatogram of Sephadex G-200 of the postmicrosomal supernatant was similar to that of the water-soluble fraction. The same two forms of enzyme were detected and only the activity in the flow-through fraction was activated by EDTA. The activity in the microsomal fraction was only 1% of that detected in the postmicrosomal supernatant. The enzyme involved in the microsomal fraction was not activated as much as that in the flow through fraction. These results suggest that the complex form is also located primarily in the postmicrosomal supernatant.

*Gel filtration of the water-soluble fraction on Sepharose 4B*

To determine the molecular weight of the complex form of enzyme, the water-soluble fraction was applied to Sepharose 4B. Fig. 5 shows the elution pattern. The first peak of the glutathione reductase activity, which corresponded to its complex form, was rather broad and these fractions were activated by EDTA. The second peak of the activity was identified as its free form, since the activity was not enhanced by EDTA and a molecular weight of about 100 000 was calculated using ferritin as a marker protein. From this elution profile, the molecular weight of the complex form was estimated to be 700 000–3 000 000.

*Sucrose density gradient centrifugation of the water-soluble fraction*

The elution profiles of the two forms of enzyme clearly indicated that they differ appreciably in molecular weight. This may allow their separation by linear

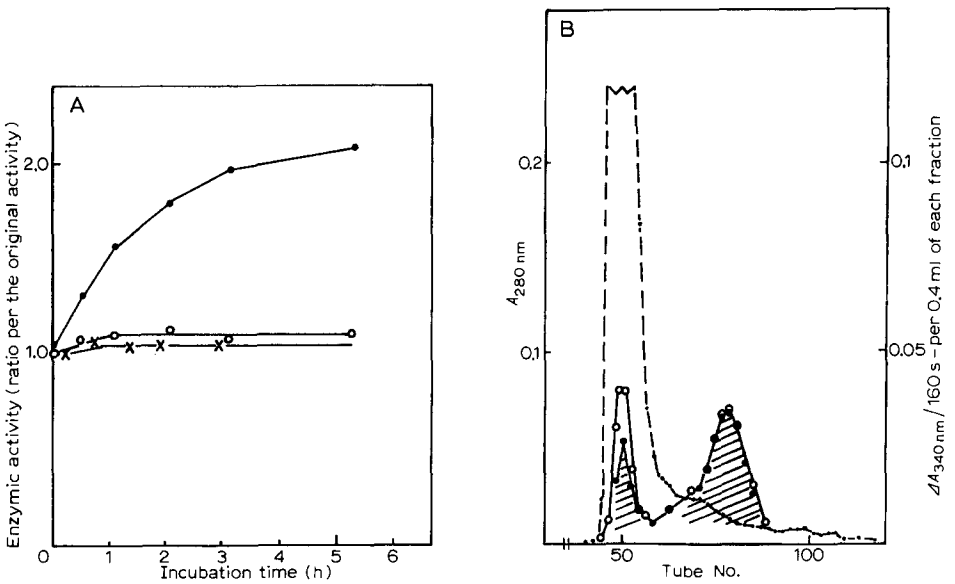


Fig. 4. (A) Activation by EGTA of glutathione reductase activity in G-200 flow-through fraction. Incubation was carried out in 5 mM EGTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C. ●—●, G-200 flow-through fraction (*Anthocidaris*); ○—○, enzyme fraction retarded through G-200 column (*Anthocidaris*); ×—×, purified enzyme (*Hemicentrotus*). (B) Rechromatogram of G-200 flow-through fraction treated with EGTA. The G-200 flow-through fraction (*Anthocidaris*) was incubated in 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C for 6 h and concentrated by a collodion bag. 6 ml of the concentrated sample (19 mg protein) were applied to a G-200 column (3 cm × 68 cm). Each fraction of 4.3 ml was analysed for protein and glutathione reductase activity. ·---·, absorbance at 280 nm; ●—●, glutathione reductase activity before incubation with EDTA; ○—○, glutathione reductase activity after incubation with 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C for 7–8 h.

sucrose density gradient centrifugation. Fig. 6 shows the sedimentation profile of the water-soluble fraction. Although the activity was expected to be separated into two peaks, on the contrary, only one peak was observed. The peak corresponded to about 6 S, and the fraction was activated about 20% by EDTA. This result meant that the sedimentation pattern of the complex form of enzyme is similar to that of the free form of enzyme. In order to ascertain this fact, the G-200 flow-through fraction and the G-200 free-enzyme fraction were separately subjected to sucrose density gradient centrifugation (Fig. 7). In both cases, only one activity peak was observed at an identical sedimentation value. However, only the G-200 flow-through fraction revealed high accessibility to EDTA. This result confirmed that the sedimentation behavior of the complex form is identical with that of the free form of enzyme under the present experimental conditions.

Since EDTA or EGTA activated the complex form of glutathione reductase by releasing free enzyme, the activation mechanism was supposed to involve chelation of divalent cations which make association of the enzyme to an inhibitor possible. A series of experiments was designed to recombine them by adding $Ca^{2+}$, $Mg^{2+}$ or $Zn^{2+}$ after their dissociation. However, no recombination, that is, no inhibition of once-activated enzyme, has so far been observed.

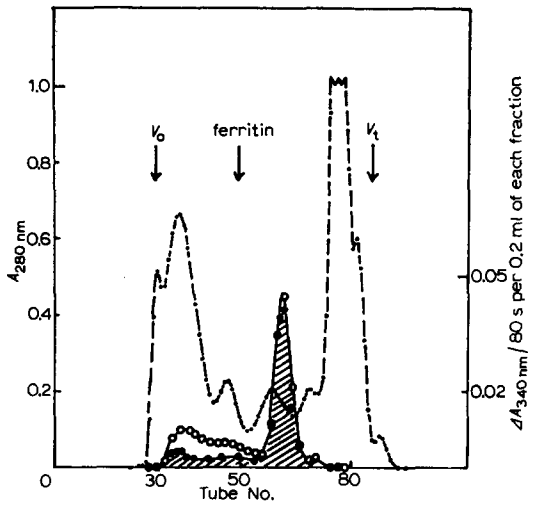


Fig. 5. Chromatogram of the water-soluble fraction on Sepharose 4B. 5.5 ml of the water-soluble fraction (26 mg protein, *Anthocidaris*) were applied on a Sepharose 4B column (2.4 cm × 55 cm). Elution was performed by 10 mM potassium phosphate buffer (pH 7.2). Each fraction of 3.3 ml was analysed for protein and enzymic activity. The void volume ($V_0$) and the column volume ($V_t$) were indicated by the arrows. Ferritin (Nutritional Biochemicals Co.) was used as a marker protein. ·---·, absorbance at 280 nm; ●—●, glutathione reductase activity before incubation with EDTA; ○—○, glutathione reductase activity after incubation with 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C for 16–17 h.

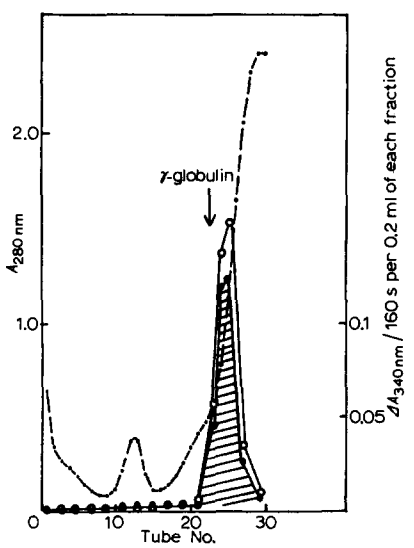


Fig. 6. Sucrose density gradient centrifugation of the water-soluble fraction. The centrifugation was carried out at 24 000 rev./min for 18 h at 0–3 °C using a Hitachi 65P ultracentrifuge (RPS 25 rotor). 1 ml of the water-soluble fraction (5.8 mg protein) was applied on a linear sucrose density gradient (5–20%, w/v) containing 10 mM potassium phosphate buffer (pH 7.2). γ-Globulin (Armour) was centrifuged in a separate tube for a standard. ·---·, absorbance at 280 nm; ●—●, glutathione reductase activity before incubation with EDTA; ○—○, glutathione reductase activity after incubation with 5 mM EDTA containing 0.2 M potassium phosphate buffer (pH 7.2) at 25 °C for 10–12 h.

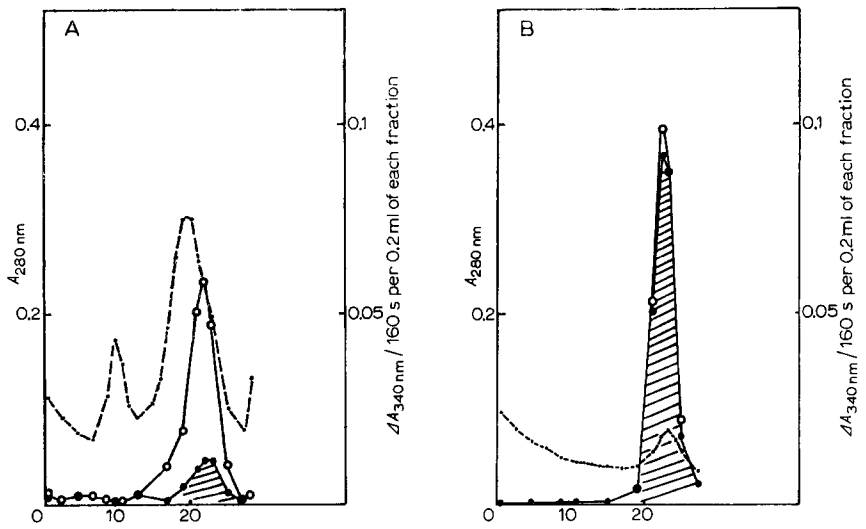


Fig. 7. Sucrose density gradient centrifugation of G-200 flow-through fraction and G-200 free-enzyme fraction. The centrifugal conditions were the same as those shown in Fig. 6. (A) 1.5 ml of the G-200 flow through fraction concentrated by ultrafiltration (Dia Flo XM 50 Amicon Co.), 8.2 mg protein. (B) 1.5 ml of the G-200 free-enzyme fraction, 0.81 mg protein; ·---·, absorbance at 280 nm; ●—●, glutathione reductase activity before EDTA-treatment; ○—○, glutathione reductase activity after EDTA-treatment, incubation for 24–26 h.

## DISCUSSION

The observation that the activity of glutathione reductase fluctuates, being closely associated to the cleavage cycle of sea urchin embryos, suggests some biochemical roles of glutathione in dynamic aspects of cell division. Assuming that glutathione reductase is a limiting factor for the rate of GSSG reduction, the increase of the enzyme activity reflects the increase in the reduction rate of GSSG. Nevertheless, the amounts of GSH and GSSG are stationary during early cell division cycles of the sea urchin embryo [1, 2]. These facts suggest that the increase of the glutathione reductase activity is accompanied by an increase in the rate of GSH consumption. Some rather strong GSH oxidizing activities were preliminarily found in the water-soluble fraction of the sea urchin embryo, namely, GSH-disulfide transhydrogenase (GSH:disulfide oxidoreductase, EC 1.8.4), GSH dehydrogenase (GSH:dehydroascorbate oxidoreductase, EC 1.8.5.1) and GSH peroxidase (GSH:$H_2O_2$ oxidoreductase, EC 1.11.1.7). These activities might fluctuate being associated with the fluctuation of the glutathione reductase activity.

–SH groups are involved in many enzymic reactions. In the sea urchin egg especially, a transhydrogenase which has been considered to be closely associated with division activity is activated by GSH [7,8]. The fluctuation of the glutathione reductase activity may also mean a fluctuation in the activities of some of the –SH enzymes.

Hosoda and Nakamura [9] demonstrated that in Ehrlich ascites tumor cells the activity of the hexose monophosphate pathway is largely dependent on the availability of $NADP^+$, which is generated through the glutathione reductase reaction coupled with glutathione peroxidase. Since the increase of the glutathione reductase activity

may cause the increase in the rate of $NADP^+$ regenerated, increase in the activity of the hexose monophosphate pathway may support the supply of materials for DNA and RNA synthesis. The timing of the increase in glutathione reductase activity coincides well with the peak of DNA synthesis in the cell cycle of the sea urchin egg [10].

Since the activities in each sample obtained at various stages of the cell division cycle, when activated through EDTA- or EGTA-treatment, reached a level identical with each other, it was suggested that the quantity of the glutathione reductase does not fluctuate during the division cycle in early development and that some inhibitory mechanism contributes to the regulation. Ordinarily, EDTA or EGTA quickly releases enzyme inhibition produced by metal ions, but a long incubation was necessary to dissociate the complex form of glutathione reductase. It is, therefore, probable that a binding site of glutathione reductase with some inhibitory factors is not located on the surface of the complex so that it is not easily attacked by the chelating reagents. Another possibility is that the activation may be due to a slow hydrolytic breakdown of the complex, through the action of contaminating cellular enzymes which might be activated by EDTA or EGTA.

D'Alessandri [11] demonstrated in human erythrocytes that the glutathione reductase activity increased by up to 175% in stored blood samples or in stored haemolysates. It was also observed in the sea urchin embryo that the glutathione reductase activity in a stored water-soluble fraction increased up to 130% during a week at 0 °C. The mechanism of this increase in the activity during a long storage may also be elucidated in terms of dissociation of such a complex.

The inhibition of glutathione reductase activity through the formation of the complex was not complete. The enzymic activity seems to be inherent in its complex form, in a range less than 25% of that of its free form (Fig. 7A). The formation of the complex could be responsible for the regulatory mechanism, although it has not been possible so far to reconstitute the complex in vitro. The present results, however, suggest a possible regulatory mechanism in which a part of glutathione reductase in the sea urchin embryo is converted to the complex and the shift of the association–dissociation equilibrium of the complex reflects the fluctuation of the glutathione reductase activity during the division cycle.

Although the molecular weight of the complex was estimated to be 700 000–3 000 000, the sedimentation pattern on sucrose density gradient of the complex was similar to that of the free form of enzyme under the present experimental conditions. This strange result was elucidated only on the assumption that the inhibitor has a high partial specific volume, which was estimated to be 0.94–0.96 (see Appendix). This high value suggests that the inhibitor may be a lipoprotein. However, a final conclusion on this point must await further purification and characterization of the complex.

## APPENDIX

When partial specific volumes of the free enzyme, the complex form and the inhibitor are defined as $\bar{v}_0$ (0.75 [12]), $\bar{v}_c$, and $\bar{v}_i$, respectively,

$$\bar{v}_c = \bar{v}_i - \frac{\bar{v}_i - \bar{v}_0}{k} \tag{1}$$

where $k$ = molecular weight of the complex form/molecular weight of the free enzyme.

Assuming the complex form is globular, the sedimentation coefficient of the complex form can be calculated from the following equation.

$$S_c = k^{\frac{2}{3}} \left(\frac{\bar{v}_0}{\bar{v}_c}\right)^{\frac{1}{3}} \left(\frac{1 - \bar{v}_c}{1 - \bar{v}_0}\right) S_0 \qquad (2)$$

where $S_0$ represents $S_{20,w}$ of the free enzyme (6 S) and $S_c$ is $S_{20,w}$ of the complex form. Following the method reported by Martin and Ames [13], the magnitude of sedimentation of particles having various partial specific volumes, was calculated as a functipn of $S_{20'w}$ of those particles under the present experimental conditions. The relationship is shown in Appendix Fig. 1. From the values of $\bar{v}_c$ and $S_c$, which were obtained from Eqns 1 and 2, $\bar{v}_1$ of 0.94–0.96 satisfies the present experimental result.

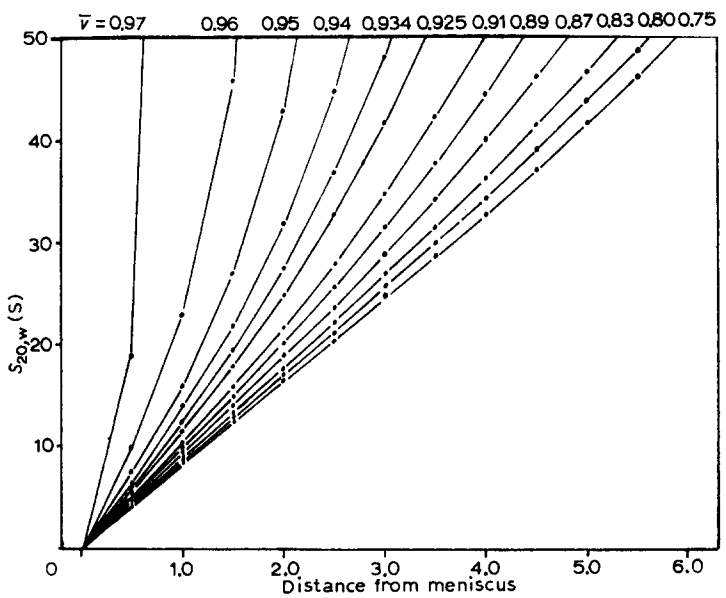


Fig. 1. Theoretical sedimentation magnitude of particles having various partial specific volumes ($\bar{v}$) as a function of $s_{20,w}$ in a 5–20% (w/v) sucrose gradient at 0 °C, when the centrifugation was carried out at 24 000 rev./min for 18 h using a Hitachi 65 P ultracentrifuge (RPS 25 rotor). Distance in cm.

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REFERENCES

1 Neufeld, E. F. and Mazia, D. (1957) Exp. Cell Res. 13, 622–624
2 Sakai, H. and Dan, K. (1959) Exp. Cell Res. 16, 24–41
3 Fredborg, A. and Lindahl, P. E. (1970) Exp. Cell Res. 59, 452–456
4 Hinegardner, R. T. (1969) Biol. Bull. 137, 465–475
5 Ii, I. and Sakai, H. (1974) Biochim. Biophys. Acta 341, 141–150

6 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
7 Sakai, H. (1965) Biochim. Biophys. Acta 102, 235–248
8 Sakai, H. (1967) J. Biol. Chem. 242, 1458–1461
9 Hosoda, S. and Nakamura, W. (1970) Biochim. Biophys. Acta 222, 53–64
10 Suzuki, N. and Mano, Y. (1973) Dev. Growth and Differ. 15, 113–123
11 D'Alessandri, A. (1969) Helv. Med. Acta 35, 118–123
12 Mavis, R. D. and Stellwagen, E. (1968) J. Biol. Chem. 243, 809–814
13 Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379