EFFECTS OF MERCAPTOETHANOL ON THE FURROWING CAPACITY OF ARBACIA EGGS [1]

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In recent years there have been several comprehensive reviews concerning the mechanisms of cell division (Gross, 1960; Mazia, 1961; Levine, 1963). However, the nature of the structural proteins believed to be involved in cytokinesis is still to be elucidated. Possibly, the answer to what mechanisms are responsible for cytokinesis could be clarified by an understanding of the role thiol groups play and their relationship to sol-gel reactions which accompany cytokinesis.

Thiol groups are known to play an important role in mitotic formation (Mazia, 1958; Mazia and Zimmerman, 1958). Inhibiting the thiol groups of cleaving cells with sulfhydryl inhibitors (salyrgan and p-chloromercuribenzoate) was found to block cytokinesis (Zimmerman *et al.*, 1957). Furthermore, the inhibition of the thiol groups is related to a weakening of the cortical plasmagel of the egg. The fluctuation in the —SH content of contractile thread models prepared from sea urchin eggs has also been studied with respect to elucidating the mechanisms of division (Sakai, 1962a, 1962b).

Experiments employing mercaptoethanol, a substance having a readily available source of thiol groups, have demonstrated that gelated structures within the cell, such as the mitotic apparatus (Mazia and Zimmerman, 1958), and the plasmagel of the amoeba (unpublished data), are markedly altered by the addition of mercaptoethanol. Since it has been proposed that the gelation reactions in the plasmagel are responsible for cytokinesis, mercaptoethanol, presumably by interfering with sol-gel reactions, should alter the structural characteristics of the plasmagel and thus affect cell division.

One tool that has been found to be useful in evaluating the effects of chemical agents on cell division is hydrostatic pressure. High pressure tends to solate the cortical plasmagel of cleaving eggs. Earlier studies (*cf.* Marsland, 1956) have shown that the structural characteristics of the cortical plasmagel are directly related to the cleavage capacity of the cell. Thus, the application of hydrostatic pressure blocks cell division. Since chemical agents may modify the structural gel components and consequently the cleavage capacity of the cell, these effects may be quantitated by measuring the pressure values essential to block division.

The present study was designed to investigate the effects of mercaptoethanol on the cleavage capacity of cleaving marine eggs. In addition, in order to establish

[1] The work was supported in part by Grant GM 07157-04, 05 from the Division of General Medical Sciences, United States Public Health Service.

[2] Part of this work was conducted while the investigator was affiliated with the Department of Pharmacology, Downstate Medical Center, State University of New York.

what effects thiol groups have on gelation reactions in the cell, pressure-centrifuge experiments were conducted on the cortical plasmagel of fertilized eggs.

Materials and Methods

Living material. Eggs from *Arbacia punctulata* were obtained by means of 0.5-ml. intracoelomic injection of 0.53 *M* KCl. The shed eggs were washed three times by decantation with filtered sea water at 20° C. The sperm were obtained from excised testes and stored as "dry sperm" at 4° C.

Pressure apparatus. The microscope pressure chamber, patterened after one designed by Marsland (1950), with certain modifications, permits cells to be observed at magnification up to 600 times while being subjected to pressure treatment. Pressure was built up by means of an Aminco pressure pump at the rate of 5000 lbs./in.^{2}/second. The pressure was released almost instantaneously by means of a needle valve.

The centrifugal studies were conducted with a pressure-centrifuge head similar to one designed by Brown (1934). This pressure chamber contains two compartments, a control compartment and a pressure compartment. Thus, the experimental and control eggs can be simultaneously centrifuged. Centrifugal forces as high as 33,000 *g* were attained within a few seconds by means of a modified Dumore motor with an appropriate pulley system.

The microscope-pressure chamber and pressure centrifuge are mounted in a specially designed temperature chamber. The temperature control housing permits the temperature to be maintained at any point between −5° and 60° C. with a maximum internal variation of ±0.3° C.

Immersion procedure. At appropriate times (20–40 minutes) after insemination, the fertilized eggs were placed into a solution of mercaptoethanol at the desired concentration 0.01–0.075 *M*. After a 20-minute incubation in mercaptoethanol the eggs were subjected to centrifugal treatment. Centrifugal displacement of pigment vacuoles was established at a magnification of 440 ×.

In establishing the cleavage capacity of fertilized eggs, the cells were immersed in the mercaptoethanol 20 minutes prior to the expected time of furrowing. Subsequently, the cells were placed in the microscope-pressure chamber, and at the time of incipient furrows they were subjected to the desired pressure.

Chemicals. The 2-mercaptoethanol ($HSCH_2CH_2OH$) was obtained from Eastman Organic Chemicals, Rochester, New York. Fresh solutions of mercaptoethanol in sea water were prepared daily and equilibrated at the desired temperature prior to use.

Results

Preliminary observations. In general, the data summarized in Table I are in good agreement with the earlier observations of Mazia and Zimmerman (1958). It appears, however, that the *Arbacia* eggs are slightly less sensitive to mercaptoethanol than the eggs of *Strongylocentrotus purpuratus*. When fertilized *Arbacia* eggs are placed into a blocking concentration of mercaptoethanol (0.075 *M*) prior to metaphase, the subsequent division is blocked. However, if the cells are placed into the same concentration of mercaptoethanol after metaphase, the cells divide, but do not progress past the two-cell stage. A decrease in the concentration of

mercaptoethanol permits the cells to develop to more advanced stages, but they are subsequently blocked. When the cells are placed in 0.025 *M* mercaptoethanol, 20 minutes after insemination, the first division is delayed 2–9 minutes, and development is blocked at the two-cell stage. However, at lower concentrations, 0.01 *M*, the cells develop at a rate very close to that of the controls; occasionally, the first division is delayed up to four minutes, but in the majority of the treated cells, there is no delay. At 0.01 *M*, the embryos proceed to the blastula stage at a normal rate.

TABLE I

The effects of various concentrations of mercaptoethanol on developing Arbacia punctulata

Eggs immersed in mercaptoethanol 20 minutes after insemination	Stage of development							
Mercaptoethanol concentration	57 min. after insemination		75 min. after insemination		95 min. after insemination		20 hours after insemination	
	% cleaved	Stage	% cleaved	Stage	% cleaved	Stage	% cleaved	Stage
0.075 *M*	0	No division	0	No division	0	No division	0	No division
0.025 *M*	7	2-cell	98	2-cell	98	95% 2-cell 3% 4-cell	95	80% 2-cell 15% 3–4-cell
0.010 *M*	60	2-cell	92	2-cell	97	4-cell	97	16–32-cell
Control	55	2-cell	96	2-cell	96	4-cell	96	Free moving gastrulae

Pressure-centrifuge studies. Pressure-centrifuge studies may be used as a means of studying the physical state of the structural elements concerned with division. Previous studies have demonstrated that the cortical plasmagel of fertilized eggs becomes rigid following fertilization (Marsland, 1956), and the relative centrifugal force required to displace the echinochrome pigment vacuoles in the cortex of the egg can be taken as an indication of gel strength. It is possible, therefore, to use these physical characteristics to ascertain the effects of mercaptoethanol on the plasmagel of dividing eggs.

Two concentrations of mercaptoethanol were employed in these centrifugal studies, 0.075 *M* and 0.01 *M*. The fertilized eggs were immersed in the mercaptoethanol 20 minutes after insemination for a duration of 20 minutes, at which time they were subjected to centrifugal treatment. The experimental eggs were treated with pressure, whereas the control eggs were centrifuged at atmospheric pressure. In each test, the centrifugal time (at 33,000 *g*) required to produce a standard displacement of the pigment vacuoles lying in the cortical region was taken as an index of the relative strength of the gel. The standard displacement utilized was the removal of all but 3–6 of the pigment vacuoles from cortical cytoplasm in the hyaline region of the centrifuged eggs observed at a magnification of 440 ×.

Previous studies have shown that this end-point is reproducible and may be used as an index of plasmagel strength (Zimmerman *et al.*, 1957; Marsland and Zimmerman, 1963).

As shown in Figure 1, at each of the four pressure levels studied, the gel strength of the mercaptoethanol-treated eggs is consistently lower than the control eggs. The eggs immersed in the blocking concentration of mercaptoethanol, 0.075 *M*, exhibited a structural strength 22–24% lower than that found for the non-treated controls. At the lower mercaptoethanol concentration, namely 0.01 *M*, the division was not blocked and the gel strength curve was parallel to the curve for the blocking concentration and to the control curve, lying intermediate between the two.

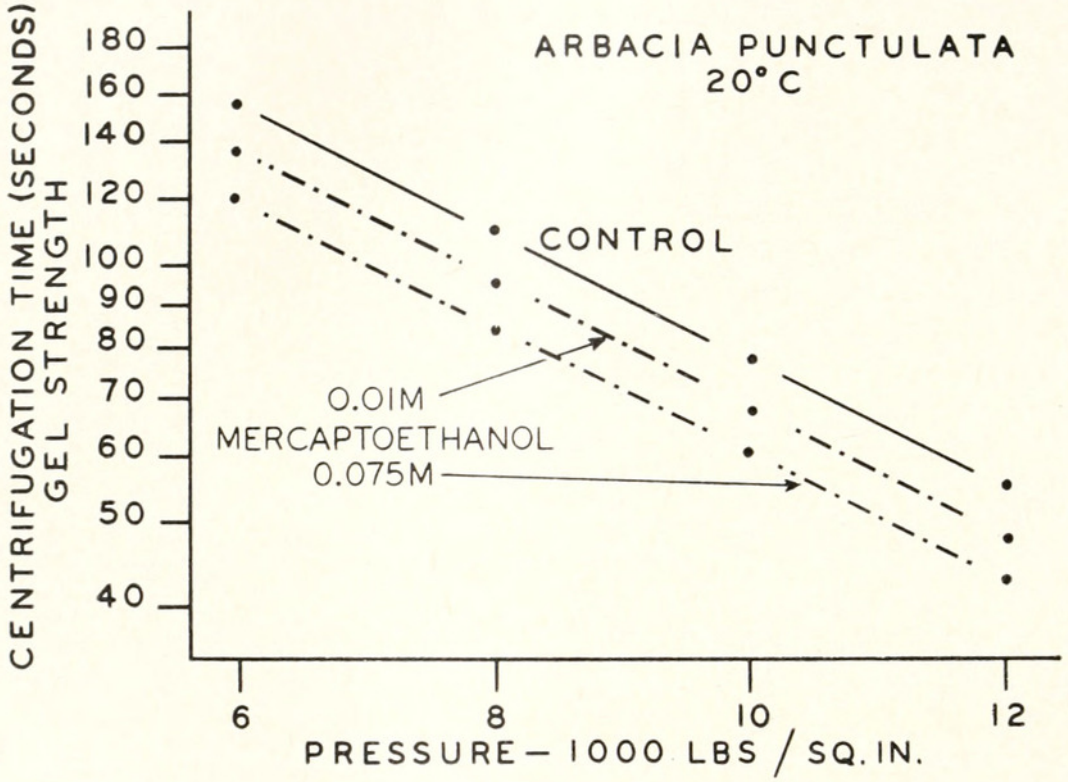

FIGURE 1. The effects of 0.01 *M* and 0.075 *M* mercaptoethanol on the gelational state of the cortical cytoplasm of fertilized *Arbacia* eggs. The gel strength is plotted as a function of pressure, at 20° C. The standard centrifugal force in these experiments was 33,000 *g*.

Each of the points plotted represents an average of 8–10 determinations. Different batches of eggs exhibit some variation in gel strength. However, in order to minimize variation, preliminary analysis permitted the selection of eggs which exhibited end-points at centrifugal times of 105–120 minutes at a pressure of 8000 lbs./in.^{2}. In all cases the slope of the curve did not vary. In those batches of eggs which exhibited high gel-strength, the slope was above that for the average. The occasional batch which exhibited lower gel-strength showed a slope slightly below the average. However, by preliminary selection of batches of eggs, variation remained ±5%. Since gel characteristics of eggs may change with increased time, we did not conduct more than ten determinations on a given batch of eggs.

Action of mercaptoethanol on cleavage capacity. Previously it was reported (Marsland, 1956; Zimmerman *et al.*, 1957) that the minimum pressure necessary to block cytokinesis may be used as an index of measuring the "furrowing or cleavage

capacity" in dividing eggs. The cleavage potential reflects the ability of the cell to divide and may be compared in normal and treated cells. Furthermore, it has been shown that the furrowing capacity is a function of the gelation state of the cortical plasmagel. Thus, it was considered essential to ascertain the effect of mercaptoethanol on furrowing capacity of these eggs.

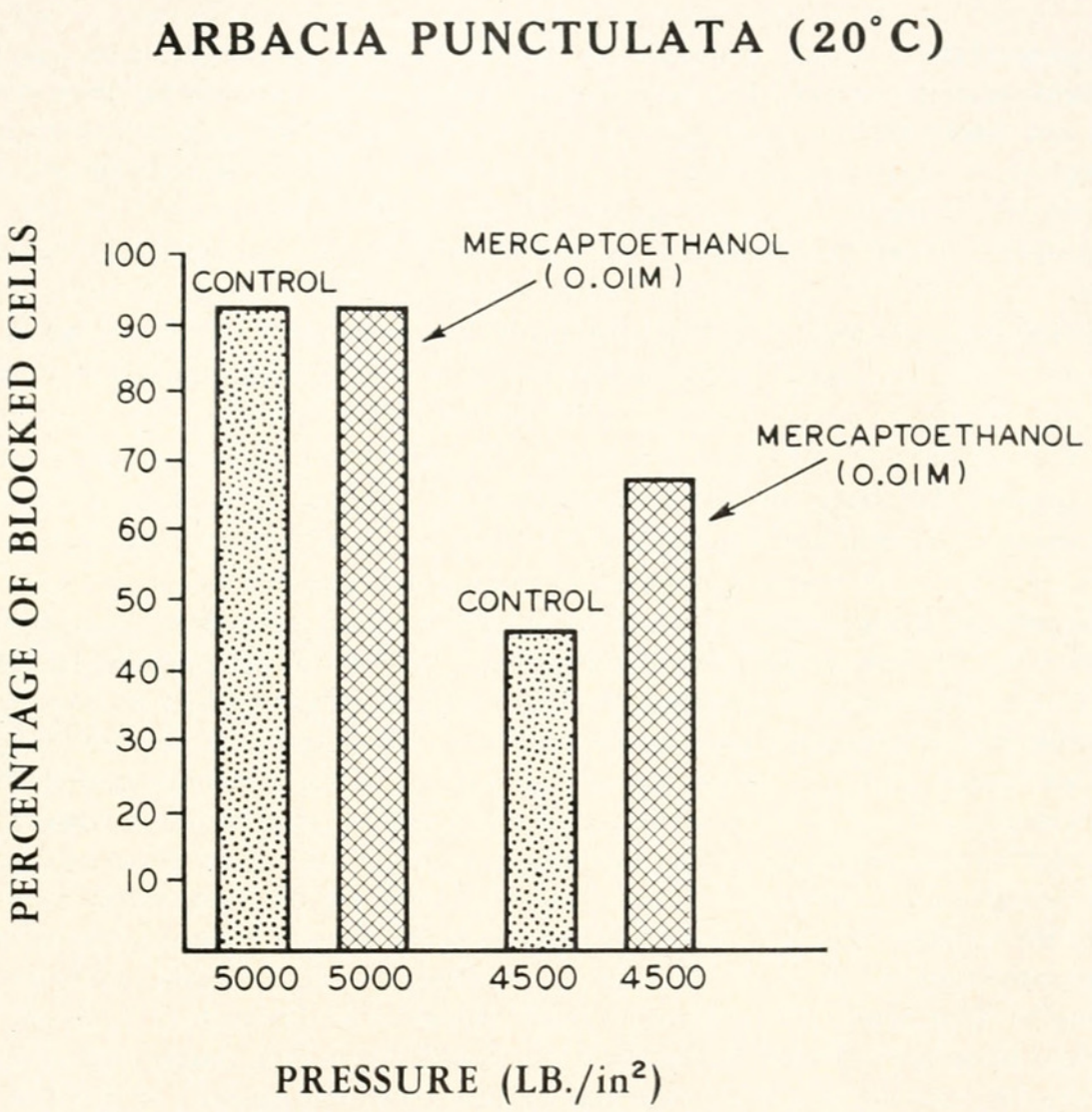

FIGURE 2. The effects of 0.01 *M* mercaptoethanol on the cleavage capacity of furrowing *Arbacia* eggs. In each experiment the percentage of blocked cells was determined after a standard compression period of 30 minutes.

The preliminary studies of varying concentrations of mercaptoethanol indicated that a concentration of 0.01 *M* has a minimal effect on first division. Therefore, this concentration was chosen for analysis of the effects of mercaptoethanol on the cleavage capacity.

The fertilized cells at 20° C. were immersed into 0.01 *M* mercaptoethanol, 20 minutes prior to the expected time of division, and subjected to a pressure of 4500 lbs./in.^{2}. After a 30-minute duration of pressure, the percentage of blocked cells was determined. In general, at this subcritical blocking pressure, 70% of the cells treated with mercaptoethanol were blocked. However, at the same pressure level

only 47% of the control eggs were blocked. Increasing the pressure to 5000 lbs./in.^{2} blocked over 95% of both the control and the mercaptoethanol-treated eggs, and no differential pressure effect could be established between the treated and control ggs at this higher pressure level (see Fig. 2).

Discussion

In general these studies demonstrate that the cortical plasmagel of the sea urchin is a thiol-sensitive structure. In addition the investigation shows that chemical substances which tend to modify the thiol-disulfide equilibrium within the cell also alter the structural characteristics of the plasmagel cortex. Furthermore, it was established that the structural changes induced by thiol compounds on the protoplasmic gel system are reflected by alterations in the capacity of the cell to furrow.

Earlier studies (Mazia, 1958; Mazia and Zimmerman, 1958) have shown that mercaptoethanol prevents the formation and produces a disorganization of mitotic structure. Blockade of cell division with mercaptoethanol inhibits the duplication of cell centers (centriole) but does not inhibit the subsequent splitting and separation of the centers that have already been duplicated (Mazia *et al.*, 1960). Although cell division and centriole duplication are prevented in the presence of mercaptoethanol, DNA synthesis is not blocked (Bucher and Mazia, 1960).

The present studies clearly show that mitotic blocking concentrations of mercaptoethanol (0.075 *M*) weaken the structural rigidity of the plasmagel. In order to establish the effect of mercaptoethanol on the cortical plasmagel in cells with functional mitotic mechanisms, lower concentrations of mercaptoethanol (0.01 *M*) were employed which did not block mitotic function. This concentration permitted measurements of the "cleavage potential" and measurements of the cortical plasmagel strength in the presence of non-blocking concentrations of mercaptoethanol. In both series of experiments the pressure required to block division, which is a reflection of the cleavage capacity, and the plasmagel rigidity were lowered in the presence of mercaptoethanol. From the earlier work conducted in this laboratory and that of Douglas Marsland, the cleavage characteristics of the cell are shown to be dependent upon the gel strength of the cortical plasmagel layer surrounding the cell.

When the thiol-disulfide equilibrium within a cellular system is disrupted, the structural as well as functional aspects of cellular activity are modified. There is strong evidence in the literature that —SH groups of constituent protein molecules may play an important role in the assembly of macromolecular complexes. The structural integrity of many different systems has been shown to be a function of sulfur linkages and the availability of —SH groups. The structural integrity of the mitotic apparatus, A-myosin and a-phosphorylase may be disrupted when the thiol contents of the systems are modified (Mazia and Zimmerman, 1958; Zimmerman, 1960, 1963; Madsen and Cori, 1956; and White *et al.*, 1957). Previously it has been reported (Zimmerman *et al.*, 1957) that interference with the plasmagel cortex of the cleaving sea urchin egg with sulfhydryl inhibitors (salyrgan, p-chloromercuribenzoate) lowers the "cleavage potential" and the gel strength of the cortical cytoplasm. The importance of the balance of thiol groups in protoplasmic gel structures has also been demonstrated with relation to the protoplasmic gel reac-

tions associated with pseudopodial stability and amoeboid movement (unpublished data). *Amoeba proteus* treated with mercaptoethanol exhibits a marked lowering of pseudopodial stability, accompanied by a weakening of the plasmagel (ectoplasm). Thus, the lowered "cleavage potential" and the decreased plasmagel rigidity in the *Arbacia* egg following mercaptoethanol treatment are to be expected if the plasmagel structure of the cell, just prior to division, is in a dynamic state shifting from thiol to disulfide bonding structures. This indeed appears to be true. Sakai (1963) has shown that the thiol content of isolated sea urchin cortices reaches a maximal —SH content at metaphase and diminishes as the cell prepares for division. By stabilizing the —SH content of the cortex of the egg with etherized sea water, cytokinesis is blocked. Following removal of ether-blocked eggs to normal sea water, the —SH content of the bound protein within the cortex diminishes as cytokinesis proceeds. Sakai (1962a, 1962b) has reported changes in contractility of thread models prepared from fertilized sea urchin eggs which relate to developmental stages. The changes in contractility of these models are accompanied by changes in the —SH content of the KCl-soluble egg proteins.

Summary

1. The fertilized eggs of *Arbacia punctulata* were immersed into various concentrations of mercaptoethanol, and the structural state of the cortical cytoplasm, as well as the "cleavage potential" of the cells, were measured.

2. Pressure-centrifuge measurements of the structural state of the cortical cytoplasm were made at various pressures (6000–12,000 lbs./in.^{2}) at 20° C., employing a centrifugal force of 33,000 *g*. A blocking concentration of mercaptoethanol, 0.075 *M*, yielded a value for the strength of the cortical gel which was 22–24% lower than that found in the non-treated controls. At a lower mercaptoethanol concentration, 0.01 *M*, division was not blocked and the gel strength curve was parallel to the curve for the blocking concentration and the control curve, but lying intermediate between the two.

3. The decrease in the gel strength was shown to be related to a decrease in the "cleavage potential." A pressure of 4500 lbs./in.^{2} applied at the time of furrowing will, in general, block about 50% of the cells from cleaving. When the eggs were pretreated with 0.01 *M* mercaptoethanol 20 minutes prior to division, there was a 24% lowering in the number of cells which completed division under pressure treatment, as compared to the non-treated pressurized controls.

4. In general, the data support the hypothesis that interference with the SH⇌S—S interaction in protoplasmic gel system is similar in both the mitotic gel system and the cortical gel system, and any interference with the delicate balance may markedly disrupt mitosis and cytokinesis.

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