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A decolorant of the sulfhydryl reagent DTNB released from live blastulae of sea urchins in saline medium

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SUMMARY

Cleaving embryos of the sea urchin *Paracentrotus lividus* exposed briefly to buffered saline release materials into the medium which will bleach a yellow mixture of DTNB and DTT [5,5′-dithiobis (2-nitrobenzoic acid) and dithiothreitol]. The decolorant activity is thermolabile and dialysable. The bleached mixture recolors rapidly on heating, suggesting the breakdown of a complex between the decolorant and the yellow DTNB ion.

The release of decolorants is maximal at the blastula stage, and in Ca-free saline, Tris-HCl pH 7. When various untreated embryonic stages are homogenized with DTNB, the assay for SH is lowest at blastula, suggesting that decolorants in the blastulae interfere with the assay.

INTRODUCTION

Blastomeric surfaces change both in quality and quantity during early embryonic development. At least two of the numerous surface functions required for normal development, membrane transport and cellular adhesion, have been shown in certain cells to depend on sulfhydryl-bearing proteins (Rothstein, 1970; Gasic & Galenti, 1966; Vacquier & Mazia, 1968). In the sea urchin, Sakai (1960) has demonstrated SH groups in the zygote cortex isolated before first cleavage. Since cell surface increases as cleavage continues, an increase in the cortical SH per embryo might be expected during early development.

We have found that live sea urchin embryos bind an increasing amount of slowly penetrating SH-binding organic mercurials, from fertilization through gastrulation, when exposed to the mercurials in a saline medium low in calcium (Wolfson, 1966). Since the mercurials bind to embryonic constituents only in low-calcium medium it might be that the mercurials compete with calcium for protein-SH at the cell surface or that the low-calcium medium alters the surface constituents, perhaps uncovering and/or releasing SH groups. Embryos exposed to isotonic saline lose Folin-positive materials, in amounts which increase steeply at concentrations of calcium less than half that of sea water (Wolfson, 1970). In attempting to measure the SH content of the Folin-positive substances, using DTNB (Ellman, 1959) we observed that the yellow DTNB ion, formed by reaction with SH compounds, was quite rapidly bleached upon addition of

certain saline extracts of live blastulae. In this paper the degree of bleaching activity released by saline-washed embryos is shown to depend upon the embryonic stage, and upon two environmental factors, pH and calcium content.

METHODS

Cultivation of embryos

Eggs of *Paracentrotus lividus* were fertilized to produce 95 % or more fertilization membranes and grown in densities of about 10000 eggs per ml in 500 ml sea water, at 18–22 °C, with rocking at 12 cycles/min.

Decoloration of DTNB

Samples of 200000 embryos at various stages were gently hand-centrifuged, and the sea water replaced with 10 ml cold saline containing 0·5 M-NaCl and 0·005 M-$CaCl_2$. After suspension in this medium, the embryos were again centrifuged and suspended in 10 ml buffered saline containing 0·5 M-NaCl, 0·001 M-Tris buffered to pH 7 with HCl. The embryos remained in buffered saline for 5 min during which they were kept in suspension by gentle pipetting. They were then centrifuged and the supernatant centrifuged at 14000 rev/min for 10 min in a refrigerated centrifuge.

The bleaching activity was measured in a standard mixture of 3 ml saline supernatant, 1 ml 0·1 M-Na_2HPO_4 and 0·1 ml 0·001 M-DTT into which 30 μl 0·01 M-DTNB were mixed at zero time in a tube in position in the spectrophotometer (Bausch and Lomb Spectronic 20). The absorbance was read at 412 nm at timed intervals thereafter.

Sulfhydryl assay of homogenized embryos

Counted embryos in suspension were washed once in cold 0·5 M-NaCl, 0·005 M-$CaCl_2$, sedimented and suspended in 2·5 ml 0·5 M-NaCl to which were added 1 ml 0·1 M sodium phosphate buffer pH 8, and 30 μl 0·01 M-DTNB. The embryos were exposed to ultrasonication for 30 sec and the resulting homogenate was centrifuged 10 min at 14 000 rev/min in a refrigerated centrifuge. The absorbance of the supernatant was read at 415 nm and compared to a standard curve for glutathione. Assays on mixtures of homogenates of embryos at different stages with glutathione added, showed the absorbance to be additive.

Ultraviolet spectra

Saline media containing decolorant from blastulae were flash evaporated at temperatures not exceeding 60 °C to approximately 1/5 the original volume. The absorption spectra of decolorant media, and similarly concentrated Tris-buffered saline, were determined spectrophotometrically (Gilford).

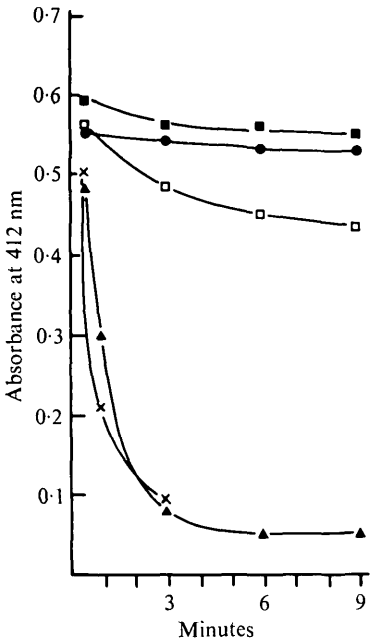

Fig. 1

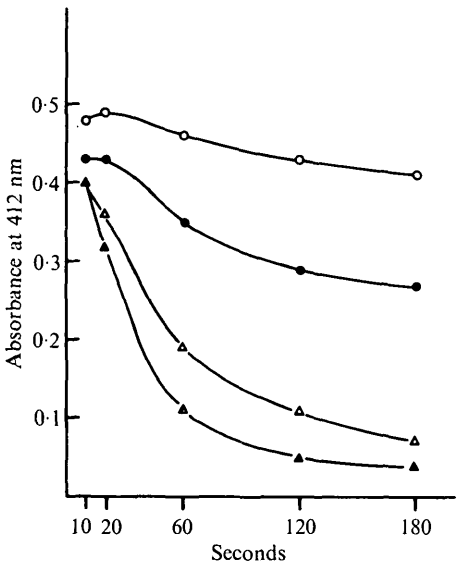

Fig. 2

Fig. 1. Decolorant released from live blastulae into buffered saline. Embryos of *Paracentrotus lividus* were cultured to blastulae, and samples of 200000 centrifuged, washed, suspended in 10 ml isotonic saline, Tris-HCl pH 7·4, for 20 min, at 18 °C. Decoloration in the supernatant after addition of DTT and DTNB was measured at 412 nm (ordinate) at intervals after mixing (abscissa). ×—×, Fresh supernatant; ▲—▲, after 24 h at 4 °C; □—□, after 5 min at 95 °C; ■—■, after 10 min at 95 °C; ●—●, and after dialysis for 8 h.

Fig. 2. Decolorant released by fertilized eggs and blastulae into Tris-buffered saline at pH 7 and 8. One-cell stage and blastulae of *Paracentrotus lividus* were treated 5 min with Tris-buffered saline, pH 7 or 8, and the decoloring activity of the supernatant was assayed as for Fig. 1. Fertilized eggs, ○—○, pH 8; ●—●, pH 7; blastulae, △—△, pH 8; ▲—▲, pH 7.

Reagents

Ellman's reagent (1959) 5,5′-dithiobis (2-nitrobenzoic acid), (DTNB), was obtained from Sigma (St Louis, Mo.). Cleland's reagent (1964) 1,4-dithiothreitol, (DTT); tris (hydroxymethyl) aminomethane, (Tris); and N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, (TES); were from Calbiochem (Los Angeles, Calif.).

RESULTS

Decoloration activity of saline supernatant

Treating blastulae of sea urchins with buffered saline solutions for periods as short as 5 min promoted the release of substances into the medium, substances which decolored the yellow DTNB ion produced after reaction of DTNB with

DTT. An active extract of blastulae bleached our standard mixture of DTNB and DTT from a maximal absorbance between 0·5 and 0·6 to a final absorbance below 0·05, in 3 min or less (Fig. 1). The rate of decoloration remains stable for 1 day at 4 °C, or for several weeks in extracts which have been frozen.

The bleaching activity was unstable to heating in a boiling water bath and disappeared after 10–15 min heating (Fig. 1). The decolorized reaction mixture recolored rapidly when heated which suggests that the basis for the bleaching action may be the formation of a complex between the heat-labile embryonic decolorant and the yellow DTNB ion.

The decoloring activity inside the dialysis bag disappeared after several hours dialysis against distilled water (Fig. 1). The activity could be recovered in the dialysate concentrated by flash evaporation and is therefore probably dependent upon small molecules.

The slope of the decoloration curves varied with the number of embryos, and with other factors discussed below, such as stage of development and pH of the medium. A practical number for producing a decoloring activity whose variations remained within the range of our assay was 200000 embryos. After the initial bleaching of a mixture of decolorant, DTNB and DTT, the addition of more DTT recolored the mixture which would often bleach a second time, but more slowly and less completely probably because of a decrease in active decolorant remaining in the mixture.

Developmental stage and release of decolorant

Unfertilized eggs released no decolorant under conditions which promoted release of decolorant by cleaving embryos, beginning with zygote stage and most readily at blastula stage (Fig. 2). Later stages of larval development were likely to release less decolorant and none was obtained from plutei. Embryos which were returned to sea water, after having released decolorant into buffered saline, developed in the same way as untreated controls.

Influence of pH and calcium on the release of decolorant

Treatment of embryos with saline buffered at pH 7 produced considerably more bleaching activity than when buffered at pH 8 (Fig. 2). Often no bleaching activity at all was obtained when alkaline saline solutions were used. Other reagents, NaOH, Na-phosphates or TES, used at the same concentration and pH as for the Tris-buffered solutions produced little or no decoloring activity, which suggests that Tris itself contributed to the release of decolorant by the embryos.

The addition of calcium to the buffered saline before treatment blocked the bleaching activity, probably by preventing release of decolorant from the embryos. To prepare decolorant supernatants, embryos were first washed in saline containing calcium at half the concentration in sea water so that some calcium remained to prevent the release of decolorant into the first wash.

Table 1. *Sulfhydryl assays using DTNB in homogenates of embryos of* Paracentrotus lividus, *at several developmental stages*

Embryos raised at 18–22 °C were sampled at the stages indicated, counted and subjected to ultrasonic disintegration in the presence of DTNB. The absorbance of the supernatant after centrifugation was read at 415 nm, and compared to glutathione standards. The results were tabulated as the mean micromoles of SH per embryo (italic value), with standard deviation from the mean (s) and number of determinations (n).

	Hours after fertilization					
	0	1–4	5–9	20–30	30–40	40–50
	Stage of development					
No. of embryos ($\times 10^3$)	Egg	Cleavage	Blastula	Gastrula	Prism	Pluteus
	μmol SH $\times 10^{-12}$ per embryo					
5–10	*13·1*	*11·1*	*10·8*	*11·5*	*14·3*	*15·6*
s	1·6		4·1	4·7	1·6	3·5
n	9	1	8	6	2	5
10–20	*9·5*	*7·9*	*8·0*	*9·1*	*10·3*	*13·8*
s	1·8	1·2	1·3	3·5	0·9	2·4
n	11	7	10	8	2	6
20–30	*7·8*	*5·8*	*6·6*	*7·5*		*12·5*
s	1·6	0·2	1·1	2·9		5·1
n	5	3	5	3		2

Blastulae thus washed then released the major portion of decolorant during the first 5 min of their exposure to Tris-buffered 0·5 M-NaCl. A second 5 min treatment with buffered saline yielded little or no additional decolorant, nor did prolonged treatments increase the yield.

Sulfhydryl assays of homogenized embryos

The amount of SH per homogenized embryo as measured with DTNB declined from unfertilized egg through cleavage to blastula, and rose gradually through gastrula, prism and pluteus stages (Table 1). When 10000–20000 embryos were assayed, the mean value for unfertilized eggs was $9{\cdot}5 \times 10^{-12}$ μmol SH per egg, which dropped to 8×10^{-12} per blastula and rose to $13{\cdot}8 \times 10^{-12}$ per pluteus. The value for blastulae is significantly lower than that for eggs ($P < 0{\cdot}05$) and for plutei ($P < 0{\cdot}01$), using the t test.

The amount of SH assayed per homogenized embryo decreased with an increase in number of embryos (Table 1), perhaps because of interference with the SH assay by materials in the embryos. The low SH values obtained from homogenates of the blastular stage would be expected if blastulae contained a greater share of interfering substances than did earlier or later stages of development. The blastular stage is that at which the live embryos released the greatest amount

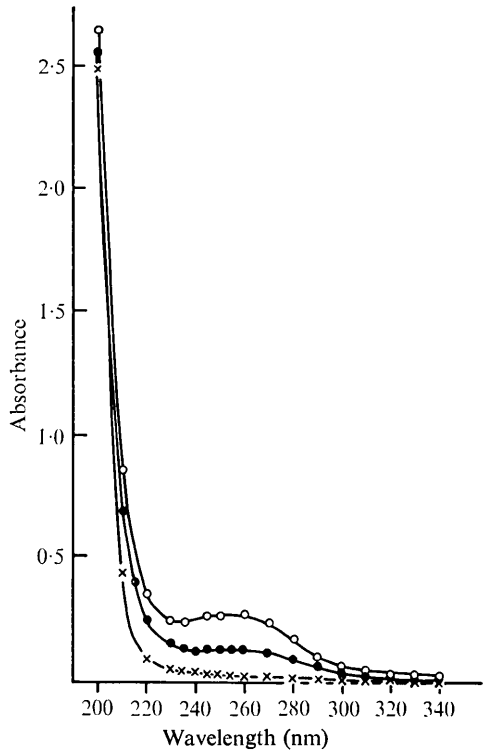

Fig. 3. Absorption spectra of materials released into Tris-buffered saline by live blastulae. Blastulae of *Paracentrotus lividus* were treated 5 min with Tris-buffered saline at pH 7 to produce a supernatant with active decolorant. A second saline wash of the same blastulae produced a supernatant with little or no decolorant. Samples of these two saline washes, and the Tris-buffered saline alone, were concentrated five-fold by flash evaporation and their absorption curve determined. ○—○, Active decolorant; ●—●, second saline wash; × — ×, Tris-buffered saline alone.

of DTNB-bleaching activity into buffered saline, and this decolorant within the embryos might well be responsible for the low SH assay of blastular homogenates, obtained using DTNB.

Ultraviolet spectrum of active decolorant solution

A concentrated solution of active decolorant released by live blastulae into buffered saline exhibited an absorption hump between 240 and 270 nm with maximum near 260 nm (Fig. 3). A second treatment of the same blastulae by a fresh solution of buffered saline resulted in lowering and flattening the absorption maximum. The concentrated buffered saline alone showed negligible absorption in this range.

DISCUSSION

When sea urchin blastulae are exposed briefly to buffered saline they release into the medium substances which bleach the yellow DTNB ion. This decolorant activity is thermolabile and dialysable. The released materials also show absorbance in the ultraviolet spectrum.

Since the addition of calcium to the saline medium before treatment of the embryos prevented the appearance of any bleaching activity after treatment, the presence of calcium either bound or in solution appears to influence physiological changes leading to the release of decolorant by the embryos. Treating the blastulae with buffered saline containing no calcium probably alters the embryonic surface so as to promote the release of decolorant from the embryos.

Calcium plays an important role both in maintaining the integrity of the cell membrane and in cell adhesion. Calcium-binding to red cell ghosts appears to depend on the integrity of membrane SH (Tolberg & Macey, 1972), and recent evidence indicates that calcium binds directly to the sulfhydryls of certain purified proteins (Toda, Kato & Narita, 1968). Acidic phospholipids constitute the main group of calcium-binding substances in cell membranes, and the amount of calcium bound to pure phospholipid monolayers is pH-dependent, dropping sharply between pH 8 and 6·5 (Quinn & Dawson, 1972). Sea urchin blastulae release a greater amount of bleaching activity in media of pH 7 as compared to 8, and this pH-dependency may be related to an effect on the membrane resulting from calcium loss from the phospholipids. The calcium-precipitable hyaline layer binding the blastomeres dissolves readily in calcium-free solutions such as the buffered saline used to provoke release of decolorant in these experiments. However, the hyaline layer (Vacquier, 1969), its protein component, hyalin (Stephens & Kane, 1970), and intercellular acid polysaccharides (Aoki & Koshihara, 1972) are all reported to be non-dialysable, and are therefore unlikely to include decoloring substances.

Developmental changes associated with the appearance of cilia and of hatching enzyme may cause the blastulae to be more sensitive to the effects of the buffered-saline treatment. The materials released by the treatment do not appear to be required for post-blastular development, since the treated blastulae developed normally upon return to sea water. Furthermore, plutei treated with buffered saline release no decolorant, an indication that these later embryos neither require nor produce the decolorants.

Embryonic constituents which are present during cleavage but absent later might function as biosynthetic repressors. In this connexion several diffusible inhibitory substances have been reported for sea urchin and amphibian embryos (Brachet, 1969; Shiokawa & Yamana, 1967, 1969). Gastrulae of the urchin *Lytechinus pictus* exposed to calcium-free sea water release materials which cause the reversible inhibition of gastrular morphogenesis (Berg & Akin, 1971). These materials have an ultraviolet absorption spectrum like that of protein,

with possibly some nucleic acid present, and are non-dialysable and heat-stable. Our extract from *Paracentrotus* is released from similar stages in similar medium and absorbs in the ultraviolet; however, the decoloring activity is heat-labile, and this lability makes it difficult to test for a long-term effect on development.

The chemical nature of the decolorant is unknown but the bleaching activity appears to depend on the formation of a complex with the yellow DTNB ion. The resulting decoloration of the yellow ion may be responsible for the low SH assays obtained on the homogenates of blastulae in these experiments. This observation draws attention to possible interference in SH assays performed on other tissues with DTNB. The bleaching activity in saline extracts of blastulae will serve to follow the unknown substance in efforts to separate it for identification.

RÉSUMÉ

Les embryons d'oursin (*Paracentrotus lividus*) traités pendant leur segmentation avec une solution saline isotonique et tamponée, libèrent des matières qui décolorent l'ion jaune formé par le réactif DTNB en présence de groupes SH. Les matières décolorantes sont thermolabiles et dialysables, et le mélange décoloré se colore de nouveau après avoir été chauffé, ce qui indiquerait la décomposition d'un complexe entre le décolorant et l'ion DTNB jaune.

Cette libération de matières décolorantes atteint un maximum au stade de blastula et ne se produit nettement qu'en milieu sans calcium et avec Tris-HCl pH 7. Les groupes SH qu'on peut doser avec le DTNB dans des broyats de divers stades embryonnaires atteignent un minimum au blastula, ce qui laisse penser à une intervention de la part des décolorants pour amenuiser le dosage des SH.

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