

## Post-fertilization changes in *Discoglossus pictus* (Anura) eggs result in the formation of a capsular chamber where the egg rotates

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**ABSTRACT** *Discoglossus pictus* is one of the few anurans with an egg where a capsular chamber forms as a consequence of fertilization; the egg with its vitelline envelope rotates in this chamber according to gravity. We investigated the formation of the capsular chamber through various experimental cytochemical and ultrastructural approaches, and found that it is the product of plug liquefaction. The plug is a lens-shaped jelly coat typical of *Discoglossus*, and covering only part of the egg animal half. About 15 min after fertilization, granular material coming from the egg enters the plug, which gradually dissolves and, once liquefied, reorganizes itself around the entire egg, thus forming the chamber. This process goes through stages of rearrangement of the 25-Å- and 250-Å-thick filaments which constitute the plug matrix. The material entering the plug derives from the exocytosis of two vacuole types, with electron transparent and granular PAS-positive contents. Liquefaction of the plug correlates with the reduction of disulfide bonds present in its matrix. Furthermore, *in vitro* tests showed that the substances released from the egg are active in selectively dissolving only the plug, and lose activity upon boiling.

**KEY WORDS:** Anuran egg, capsular chamber, jelly liquefaction, thiol groups

### Introduction

Amphibian eggs are endowed with the vitelline envelope (VE) and several jelly layers. In anurans, cortical granule (CG) exocytosis takes place a few min following fertilization. As a consequence, the VE lifts from the egg surface and is transformed into the fertilization envelope (FE) (for a review, see Schmell *et al.*, 1983). The perivitelline space enlarges and the egg becomes free to rotate in this space according to gravity. Rotation is essential for correct embryonic development. Information for dorsality resides in the vegetal and equatorial regions of the egg. If, during grey crescent formation, such regions are not positioned according to gravity, the embryo dorsal axis does not form correctly (for a review, see Wakahara, 1989).

In urodele eggs, CG's are absent, and the VE as well as the perivitelline space does not appear to change upon fertilization. The so-called capsular chamber forms from transformation of the innermost jelly layer, ( $J_1$ ) (Salthe, 1963). This change occurs independently of fertilization in *Pleurodeles*; large granules are present in  $J_1$ , which slowly dissolves upon ovideposition in the water (Jego

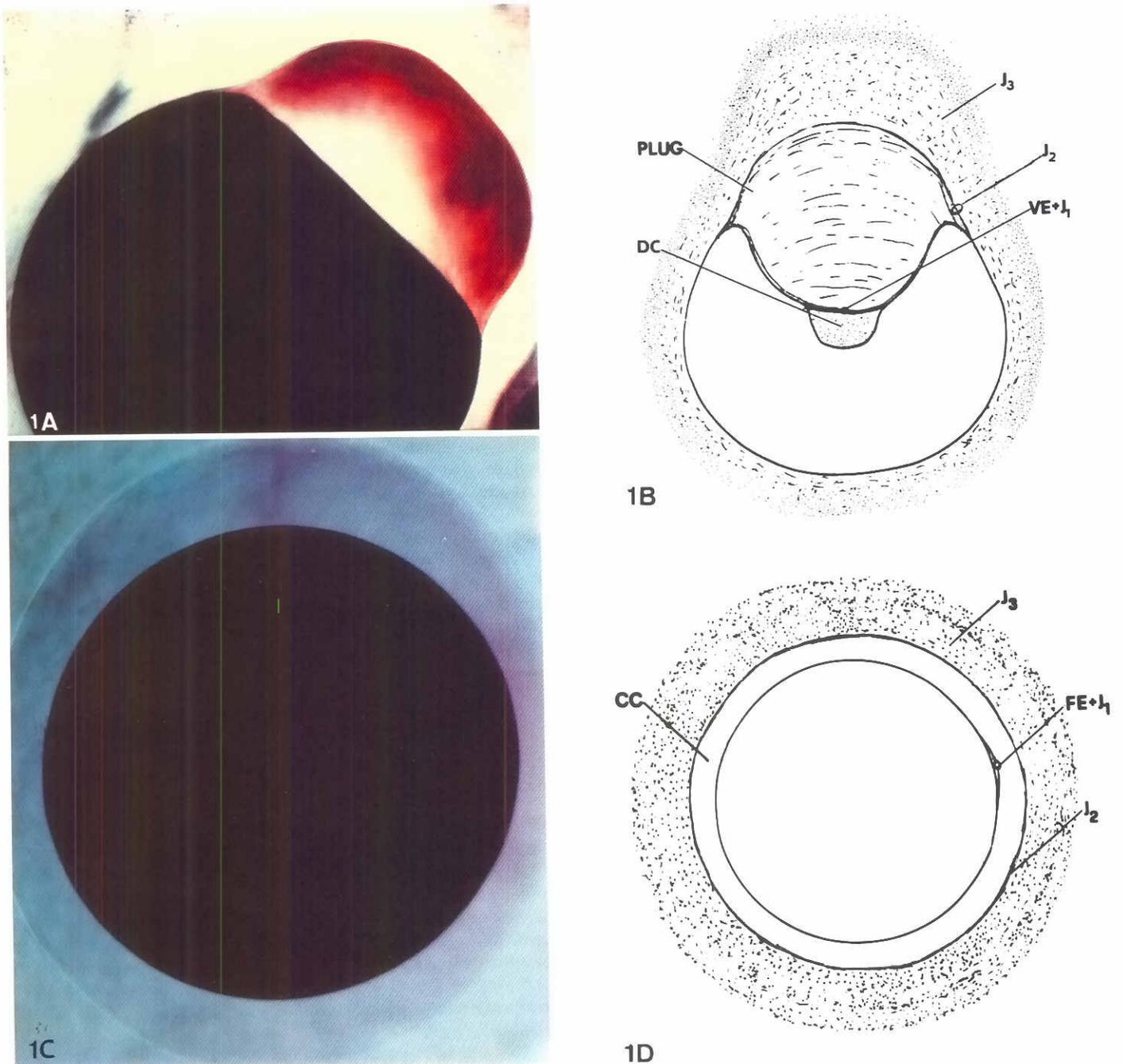
*et al.*, 1986). As a consequence of  $J_1$  transformation, the egg rotates together with its VE in the capsular chamber.

However, urodeles and anurans do not always show distinct patterns of egg investment transformation. Indeed, among anurans, capsular chambers form in the eggs of genera belonging to the same superfamily, *Discoglossidae* (*Alytes* and *Discoglossus*) or to evolutionarily distant families, *Leptodactylinae* (*Eleuterodactylus*) and *Pipidae* (*Pipa*), indicating that this process has developed independently of evolutionary trends (Wintrebert, 1928; Salthe, 1963).

The animal hemisphere of the *D. pictus* egg has a large depression, the *concavity*; at its center there is a small indentation, the *dimple*, which is filled with a fibrillar electron-dense material (Hibbard, 1928; Campanella, 1975). The central portion of the

*Abbreviations used in this paper:*  $J_1$ , jelly 1;  $J_2$ , jelly 2;  $J_3$ , jelly 3; FE, fertilization envelope; D1, dimple 1; D2, dimple 2; D3, dimple 3; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid).

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**Fig. 1.** Egg of *Discoglossus pictus* vitally stained with toluidine blue.  $J_3$ = jelly 3;  $J_2$ = jelly 2;  $J_1$ = jelly 1; VE= vitelline envelope. **(A)** Unfertilized uterine egg. The plug is purple. (x48). **(B)** Schematic drawing of a longitudinal section of a *D. pictus* egg. D= dimple. The plug is sitting at the center of the animal hemisphere, in the concavity, which is typical of the eggs belonging to the genus *Discoglossus*. **(C)** Capsular chamber in a fertilized egg. The chamber is violet and externally delimited by  $J_2$  (x50). **(D)** Schematic drawing corresponding to Fig. 1C and showing the capsular chamber. FE= Fertilization Envelope.

dimple, D1, is the site where fertilization occurs, whereas D2 and D3 are the dimple lateral walls (Talevi and Campanella, 1988). The concavity forms during the passage through the oviduct, because of the gradual deposition of the jelly plug. In oviposited eggs, the lens-shaped plug is surrounded by a thin jelly coat,  $J_2$ , and the more

conspicuous  $J_3$ . The presence of the plug at the animal hemisphere and of  $J_3$ , which is three-fold thicker on the plug, gives the egg a typical asymmetrical configuration (Fig. 1). The plug sits on  $J_1$ , which together with the VE constitutes the innermost egg envelopes (Ghiara, 1960; Denis-Donini and Campanella, 1977).

The plug has the specific characteristic of causing convergence of sperm on the dimple (Talevi and Campanella, 1988; Talevi, 1989).

At present the cytological as well as the physio-chemical basis of the capsular chamber formation is unknown in anurans.

This paper describes the formation of the capsular chamber in *Discoglossus pictus* and analyzes the factors involved in this process.

## Results

### Description of the capsular chamber formation

In *D. pictus* eggs, about 4 min after fertilization the dimple regresses, and a few minutes later, the concavity disappears (Campanella *et al.*, 1986, Campanella, 1991).

A total of sixty eggs from 15 females were inseminated and scored for these changes. While dimple disappearance can be easily determined only in dejellied eggs, the changes in the concavity can be depicted also in jellied eggs as they involve a more superficial region of the animal hemisphere (see Talevi and Campanella, 1988). The concavity regression depended upon modifications of the plug; it started 7 to 20 min after fertilization. This delay varied according to the egg clutches. The process is described in Figs. 2 and 3. Under incident light microscope, a sudden lifting could be observed at the center or the limits of the concavity (Fig. 2A,B) which extended to the rest of this region in about five min (Fig. 2C). Under the compound microscope, the first stage of this phenomenon appeared to be a sudden contraction of the concavity and a concomitant release of granular material from the egg (Fig. 2). This was followed by relaxation and lifting of the concavity and a loss of viscosity of the plug matrix. Several large globules (5-10  $\mu\text{m}$  in diameter) could be seen in the plug. They migrated towards  $J_2$ , at the plug boundary, with a velocity increasing with the loss of plug viscosity.

Plug liquefaction was accomplished in about 20 min and was also indirectly revealed by a gradual shift of the sperm bundle which had been spanning the plug since insemination (compare Fig. 2D with E).

A space, the capsular chamber, formed all around the egg because of the expansion of the plug-derived material (Fig. 1B) and changes that will be described below. The egg positioned itself in the chamber according to gravity.

The temporal relationship between egg material entering the plug and plug matrix dissolution clearly suggests that such material contains factor(s) which cause its liquefaction.

### Cytological and ultrastructural observations of egg sections

In sections of eggs fixed at early stages of the reaction (10 min after fertilization), the cortex of the concavity (see Fig. 1B) contained PAS-positive granules, about 1.5  $\mu\text{m}$  in diameter (Fig. 3A,B). At this stage, some of the granules as well as PAS-negative clouds were found outside the egg in the perivitelline space at the concavity (Fig. 3B). The latter contained a fibrillar matrix, the dimple content (see Fig. 1B), which had spread to the entire concavity as a result of dimple regression.

PAS-positive granules were not cortical granules because the latter had been exocytosed from the dimple within 2-3 min following activation (see Campanella *et al.*, 1988); this exocytosis will be here referred to as "primary exocytosis".

Ultrathin sections of eggs fixed at the beginning of the reaction

showed two types of vacuoles in the regressed dimple both at the peripheral cytoplasm and close to the plasma membrane. One type included about 0.4  $\mu\text{m}$  large vacuoles with little electron-dense content and a small invagination of the limiting cytomembrane (Fig. 4A, B). The second type included 1.3- $\mu\text{m}$ -large vacuoles with sparse fibrillar contents (Fig. 4A); because of their size and ultrastructural features, they might correspond to the PAS-positive granules. Both types of vacuoles could be seen along the whole egg periphery.

Several cases of vacuole exocytosis could be depicted due to the presence of invaginations containing a matrix dissimilar from that present on the egg surface and of hybrid membranes deriving from vacuole membranes and the oolemma (Fig. 4A,B,C) (see Campanella *et al.*, 1986). The exocytosed material was either granular (Fig. 5) or electron-transparent (Figs. 4 and 5B) and could be seen on the egg surface, in VE-J1 (Fig. 5C and D), as well as in the plug where it was concentrated in spherical, 3-4  $\mu\text{m}$  large aggregates (Fig. 5B).

In eggs fixed when plug liquefaction became evident, most vacuoles had been exocytosed. Correspondingly, the plug matrix, which was made of alternating layers of thin and coarse fibers (Fig. 5B), had profoundly changed. The coarse-fiber layers had disaggregated, the fine filament layers had wound into globules up to 10  $\mu\text{m}$  in diameter (Fig. 6), i.e., of the same size as those depicted under the compound microscope.

During this process,  $J_1$  appeared largely unaffected despite some loosening of its matrix (see Fig. 5B and D). Both  $J_2$  and  $J_3$  were morphologically unaltered. However, during the final stages of the capsular chamber formation,  $J_3$  was redistributed over  $J_2$  in a more homogeneous fashion than in the unfertilized egg (Fig. 1B), as observed in *in vivo* conditions.

In conclusion, exocytosis is involved in the plug liquefaction process and two kinds of vacuoles appear to contain factors causing this phenomenon. This exocytosis will be here referred to as "secondary exocytosis".

### Involvement of disulfide-bond reduction in plug liquefaction

It is known from laboratory practice that amphibia jelly coats are dissolved by thiols such as DTT or cysteine, because of the presence in the jelly of disulfide bonds (Gusseck and Hedrick, 1971). This observation suggests that a disulfide-bond reduction in plug proteins might be involved in plug dissolution.

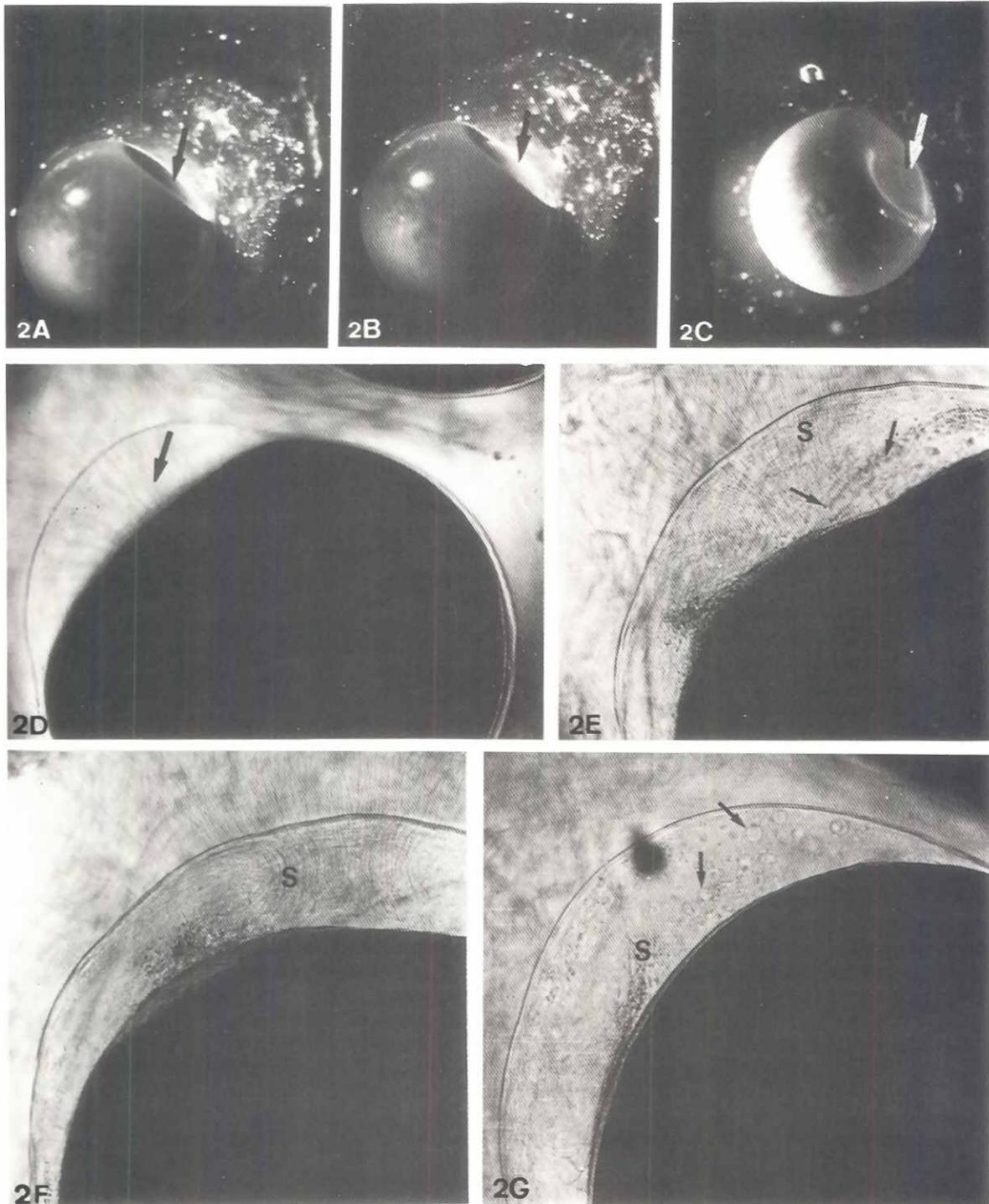
Table 1 shows the results of SH-and-S-S- content dosage of plug proteins obtained in three different experiments.

Following fertilization the content of free thiol groups became 4 to 10 times higher, whereas the content in disulfides dropped in a similar way. Similar trends were obtained when the concentrations were referred either to the egg or protein concentration.

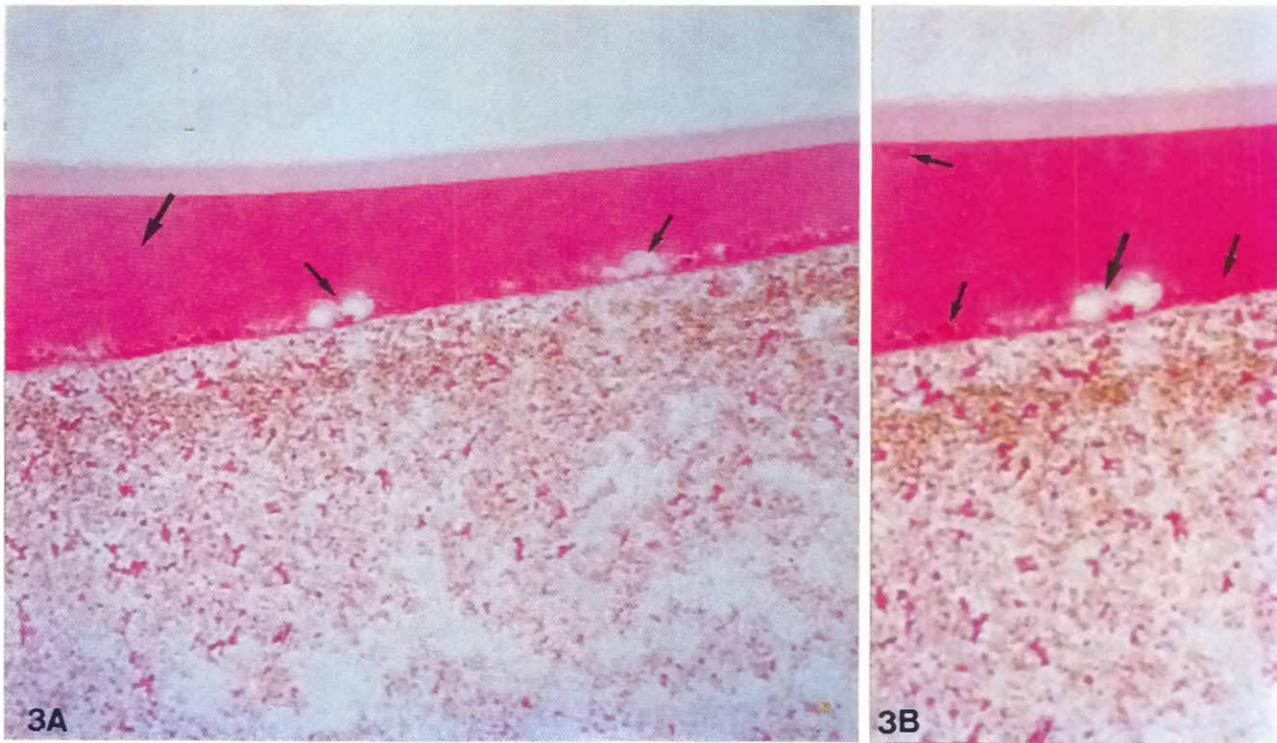
Sonication of plugs completely solubilized the proteins of fertilized eggs, whereas parts of the plug proteins deriving from unfertilized eggs were less soluble; the less soluble proteins were richer in -SH groups. The data in Table 1 concern the total protein fraction, and are very similar before and after TCA precipitation; the contents in thiols or disulfides of the supernatant after TCA precipitation is below detection limits (<0.5nmol).

Denaturation with urea had a minor effect on free thiol group concentration, whereas it allowed the determination of a larger number of disulfide bonds.

The plug might be contaminated by a small amount of  $J_3$  during manual dissection. The determination of thiol and disulfide content of  $J_3$  was performed on unfertilized and fertilized eggs. Results did not change following fertilization; the amount of free thiol groups



**Fig. 2. Eversion of the concavity.** (A-C) Sequence depicting the eversion of the concavity at the animal hemisphere (arrow at incident light during the capsular chamber formation. (x20). (D) Freshly inseminated eggs, under the compound microscope. The arrow indicates the spermatozoa converging on the plug. (x46). (E) Egg 18 min after fertilization. Granular material (small arrow) enters the plug, which starts liquefying. S= spermatozoa. (x55). (F) Five min from the onset of the reaction, plug liquefaction takes place, as indirectly evidenced by the sperm (S) shift, the expansion of the space occupied by the plug and the changes in the plug matrix. (x55). (G) At the final stage of liquefaction herein indicated, large globules can be seen in the liquefied plug (small arrow). S= spermatozoa. (x55).



**Fig. 3. Resin-embedded sections stained with PAS. (A)** Egg at the onset of the plug liquefying reaction. The extracellular material indicated by the arrow is the dimple content (DC) which occupies most of the concavity, i.e., an area larger than the dimple of unfertilized eggs. The DC is very intensely PAS-positive. On the egg surface (small arrows) one can see several unstained areas which depict PAS-negative material derived from the egg. In the concavity, such granules were also found in the bulk of the cytoplasm for 1/3 of the egg depth. On the periphery of the rest of the egg, PAS-positive granules were located in the cortex as well as in a 200- $\mu$ m-thick belt (data not shown) (x320). **(B)** At a larger magnification, PAS-positive granular material can be seen in the egg peripheral cytoplasm, on the egg surface and in the DC (small arrows). The arrow indicates PAS-negative material. (x1000).

was negligible ( $<0.15$  nmol/egg;  $<5$  nmol/mg protein) and the concentration of disulfides was about 1 nmol/egg (about 40 nmol/mg protein). Therefore, contamination of plugs by  $J_3$ , if present, did not alter the experimental data in Table 1. These data indicate that plug liquefaction correlates with disulfide-bond reduction. The latter does not involve the most conspicuous egg jelly layer,  $J_3$ .

#### In vitro tests

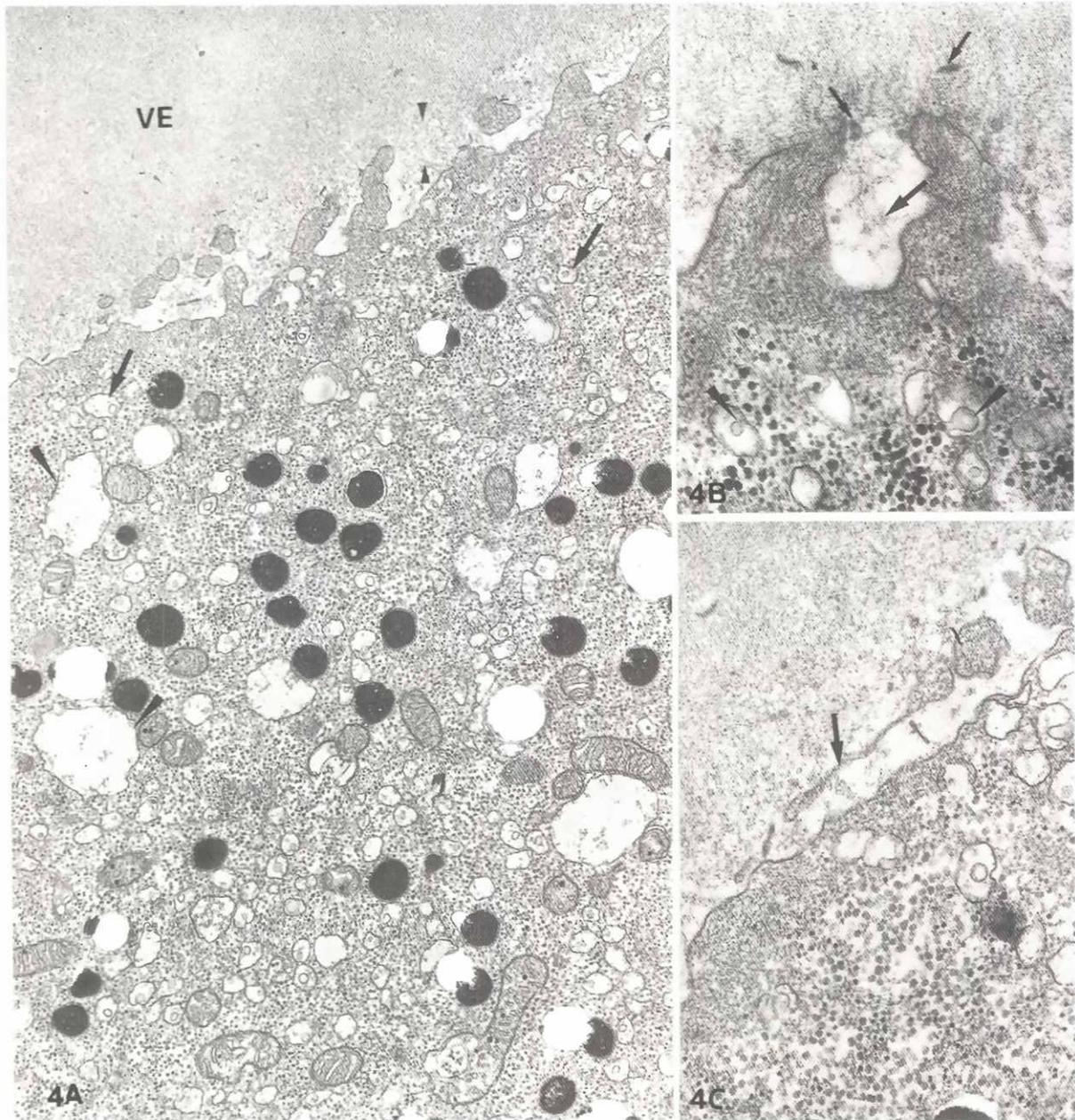
In order to ascertain the biological activity of the material exocytosed from the egg, the following experimental procedure was performed.

Clutches of eggs were inseminated and scored for timing the arousal of plug liquefaction. Thereafter sets of 10 eggs were inseminated and readily immersed in 5 mM dithiothreitol (DTT), 0.1 M NaCl, 0.01 M Tris-HCl buffer, pH 8.0. The outermost jelly layers were removed in about 5 min by chemical treatment and with the help of fine forceps. Following several rinses, the eggs, endowed only with the VE-J1 complex, were placed in fresh 1/10 Ringer before the secondary exocytosis had occurred. Fifty min later, the Ringer (about 150  $\mu$ l) where the eggs were soaking (*egg Ringer*) was transferred to a new dish. A freshly dissected plug of unfertilized egg, partially surrounded by VE-J1 and  $J_2$ , was immersed in the egg Ringer together with a piece of  $J_3$  as control and observed for changes in its matrix. In all the experiments (10 tests), the plug dissolved about 30 min later, while the other jelly coats did not

change. This indicates that the exocytosed material present in the egg Ringer has the specific ability to liquefy only the plug matrix. Egg Ringer collected upon soaking of the eggs 20 min following insemination was not effective in plug dissolution, while this ability was found in egg Ringer collected between 45-60 min after insemination, indicating that under the experimental conditions here used, it takes longer for the reaction to be accomplished (see previous paragraph) than under natural conditions.

Similar results were obtained with egg Ringer from artificially (A23187) activated eggs; when dissected plugs were challenged with egg Ringer produced by these activated eggs, they were dissolved as in the fertilization experiments. As controls, Ringer was tested, recovered either at the time of primary exocytosis (see Materials and Methods) or from unactivated eggs soaking for at least 60 min; neither sample was active. Furthermore we could determine that the egg Ringer loses its activity as a result of heating at 90°C for 5 min.

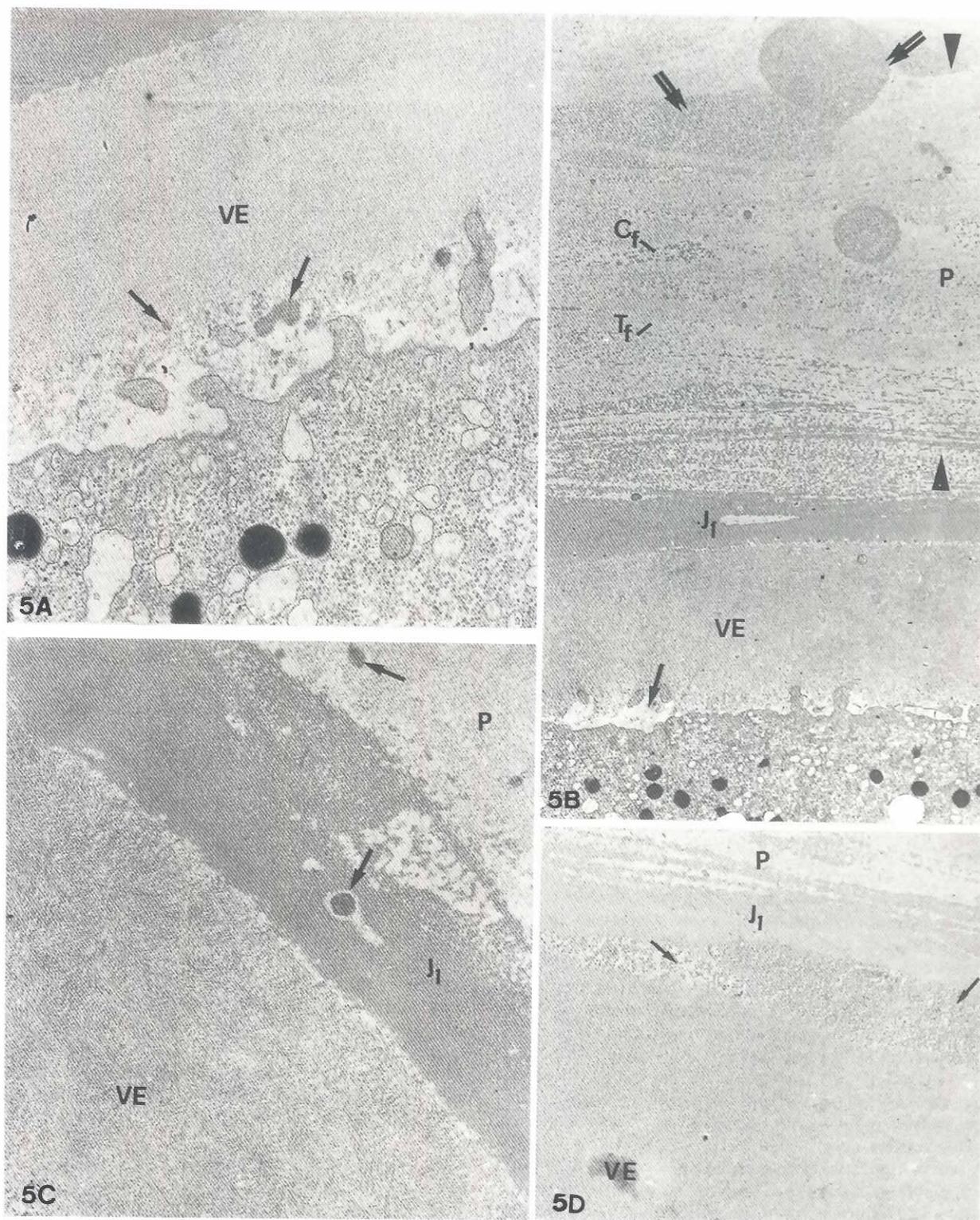
In a second group of experiments, following jelly removal, A23187 exposure, primary exocytosis occurrence and rinsing, the eggs were placed in a quartz cuvette containing fresh 1/10 Ringer and the surrounding solutions read at the spectrophotometer at 280 nm against a blank of Ringer containing an equal amount of dejellied, unactivated eggs. In the 3 experiments performed, measurements indicated that within a few minutes, an approximately ten-fold increase in absorption occurred, followed by stabilization of



**Fig. 4. Vacuoles involved in exocytosis.** (A) Two classes of vacuoles can be seen in the dimple about 12 min after fertilization. They are located at the egg periphery, and especially beneath the plasma membrane. One is 0.4  $\mu\text{m}$  large and is characterized by a small intravacuolar invagination of the limiting cytomembrane (arrows). The second vacuole is about 1.3  $\mu\text{m}$  large and has sparse fibrillar content (arrowheads). On the egg surface, hybrid membranes are present, deriving from the exocytotic process. VE=vitelline envelope typically made of interwoven fibers. A shallow perivitelline space (arrowheads) containing fibro-granular matrix was located between the VE and the egg plasma membrane. (x18,500). (B) Example of vacuole exocytosis. The invagination contains a sparse matrix (arrow) different from that present on the egg surface and similar to that in the vacuoles. Hybrid membranes deriving from the vacuole membrane and the oolemma are also found (small arrows). In *D. pictus* eggs, earlier stages of exocytosis are hardly found, as already indicated in previous papers (Talevi et al., 1985; Campanella et al., 1986). The arrowheads show internal invaginations of the 0.4- $\mu\text{m}$  vacuoles. (x21,600). (C) Electron-transparent material is observed on the egg surface where large hybrid membranes are also present (arrow). The latter can be interpreted as deriving from multiple fusions of small vacuoles. (x35,000).

these values. Fig. 7 shows the time course of this process for three different experiments, followed at 280 nm; the egg suspension was shaken before each record. Assuming an average absorption

coefficient  $A^{1\%}_{1\text{cm}}=15$  at 280 nm, about 1  $\mu\text{g}$  protein per egg was liberated in the medium after 20 min. The absorption of the solution of untreated eggs, taken as control, was almost constant for 20



**Fig. 5. Exocytosed material on the egg surface and investments.** (A) Small aggregates of electron-dense material (arrows) can be observed next to the plasma membrane. ( $\times 27,000$ ). (B) The exocytosed material is also present in electron-transparent regions (arrow) on the egg surface; in the plug (P), it is found in the shape of aggregates of fibro-granular material (double arrows). One can observe  $J_1$  tightly packed and electron-dense constituents, as well as the alternating layers of coarse (Cf) and thin fibers (Tf) of the plug. ( $\times 9,000$ ). (C and D) The exocytosed aggregates (arrows) are found in  $J_1$  and in the plug (P). In D, a large mass of electron-dense material (small arrows) is present between VE and  $J_1$ . In this layer, some loosening of its matrix occurs during plug liquefaction. (C =  $\times 40,000$ ; D =  $\times 9,000$ ).



**Fig. 6. Plug transformation during liquefaction.** About 20 min after fertilization, the plug matrix layers of coarse fibers have disaggregated; the fine filament layers have wound up into globules up to 10  $\mu\text{m}$  in diameter. S = sperm tail in cross section. ( $\times 14,000$ ).

min. The raw material extruded from the egg following activation showed a free thiol content below the detection limits ( $<0.3$  nm/egg); after TCA precipitation the supernatant had about 6 nmol-S-S per egg, the proteic fraction about 30 nmol-S-S per egg. This fraction could be cross-contaminated with residues of the dissolved plug.

## Discussion

We investigated the capsular chamber formation through *in vivo* observations, cytochemical and ultrastructural analysis as well as tests for protein and thiol group determinations. The results revealed new findings: (1) plug liquefaction occurs at fixed times following fertilization and is caused by substances released from the egg; (2) the phenomenon is temporally correlated with the exocytosis of two types of vacuoles containing at least two kinds of substances, one being PAS-positive; (3) plug liquefaction occurs through the reduction of disulfide bonds present in the plug gelatinous matrix; (4) the substances released from the egg contain components whose denaturation by heating determines a loss of the plug-liquefying activity.

Within 20 min from fertilization or activation an intraovular signal causes egg contraction and extrusion from the egg of substances which provoke plug liquefaction. The nature of the signal is unknown, but its effects can be discussed, as follows.

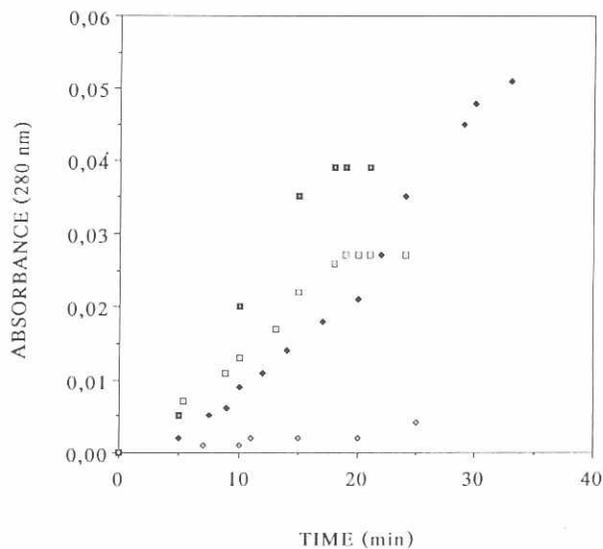
The timing of changes occurring in the plug suggests that coarse fibers are the first target of the exocytosed material, and fine filament layers get organized in large globules before dissolution. Other jelly layers do not show similar changes in plug matrix conversion. Accordingly, in *in vitro* experiments the egg Ringer does not dissolve  $J_1$ ,  $J_2$  and  $J_3$ , indicating that the process which causes

plug liquefaction is highly specific for this jelly coat.

The capsular chamber may form all around the egg because a thin layer containing the plug matrix is present over the entire egg surface (data not shown). This indicates that during the passage through the oviduct, the plug matrix is deposited all around the egg as a thin layer except for the animal hemisphere portion surrounding and including the dimple where this jelly is deposited forming the conspicuous plug. The mechanism underlying this selectivity in jelly deposition will be the subject of a separate publication.

The material released from the egg, and then found on the egg surface as well as next to both sides of VE-J1, is contained in two types of vacuoles located along the entire periphery of the egg. One class of vacuoles can be easily detected under the light microscope because of its size (about 1.3  $\mu\text{m}$ ) and PAS-positivity. In the unfertilized egg, vacuoles with similar characteristics are present in the deeper region of the peripheral cytoplasm next to the endoplasmic reticulum clusters (Campanella *et al.*, 1988). Therefore it can be inferred that, following primary exocytosis, they have reached a more superficial position. Their sparse content and size make them similar to the acidic granules of the sea-urchin egg (Lee and Epel, 1983).

The second type of vacuoles — 0.4  $\mu\text{m}$  large — is clearly detectable only in thin sections. In *D. pictus* unfertilized eggs, vacuoles similarly characterized by cytomembrane invaginations exocytose during "primary exocytosis" (Campanella *et al.*, 1986). The observed PAS-negative clouds of exocytosed material appear to correspond to the electron-transparent material observed under the electron microscope and may well derive from such vacuoles as no PAS-positivity was detected on granules smaller than 1  $\mu\text{m}$ . However, cytochemistry on thin sections is needed to draw conclusions on this point.



**Fig. 7. Absorbance time course at 280 nm of solutions containing dejellied eggs activated by A23187.** Three sets of experiments are reported. The eggs were placed in 1 ml 1/10 Ringer in the quartz cuvette after primary exocytosis had occurred. Blanks contained equal amounts of unactivated dejellied eggs. = 24 eggs; = 40 eggs; = 20 eggs; = blank of experiment.

Disulfide bridges are important intramolecular constituents of the amphibian jelly coat (Katagiri, 1963; Gussek and Hedrick, 1971). We have here shown that there is a clear increase of sulfhydryl groups in plugs examined at various stages of dissolution. Differences evidenced among the three sets of experiments may be related to the different conditions of jelly hydration, which, in turn, may be associated with seasonal conditions; eggs obtained from the ovisac in September-October are often surrounded by highly hydrated jelly. Data referring to fertilized eggs may be underestimated, because part of the dissolved plug was probably lost during collection.

The role of interchain -S-S bonds in stabilizing structural proteins and maintaining insolubility or higher levels of viscosity is well known. For example, acetylcystein liquefies mucus by acting directly on disulfide bonds of mucoproteins and thus lowering mucus viscosity.

It would be interesting to study whether the capsular chamber formation in *Pleurodeles waltii* (Jego *et al.*, 1986) shares some similarities with the process here described in *D. pictus*; the granules which dissolve in *Pleurodeles* innermost jelly layer might be active in the reduction of disulfide bonds.

The secondary exocytosis product contains the factors active in plug liquefaction; neither the egg Ringer of unactivated eggs nor the primary exocytosis product shares this activity with the egg Ringer.

As there is a precise temporal coincidence between vacuole exocytosis and the onset of plug dissolution, it can be concluded that vacuole content causes plug dissolution. However, it cannot be ruled out that other substances released from the egg through different mechanisms, may participate in this process.

Proteins probably rich in disulfide bridges are present in the "egg

Ringer". The fact that the activity of egg exudate was lost by boiling, strongly suggests that proteins are among the active factors causing plug dissolution. Studies are in progress to determine the nature of such proteins.

Early post-fertilization changes are well known to occur in the  $J_1$  of other anuran eggs following CG exocytosis at the time of fertilization envelope (FE) formation (Wyrick *et al.*, 1974; Yoshizaki, 1984, 1989; Katagiri, 1987; Bakos *et al.*, 1990). Furthermore, at advanced stages of embryonic development, the hatching enzyme is secreted by a specific region of the embryo and causes dissolution of the jelly envelope (Carroll and Hedrick, 1974; Yamasaki *et al.*, 1990). To our knowledge, the present paper is the first to describe early post-fertilization changes in a jelly layer of anuran eggs, unrelated to FE formation and involving an exocytotic process.

## Materials and Methods

Adult *Discoglossus pictus* males and females were captured near Palermo in Spring and kept in aquarium at 18°C. Gametes were collected and insemination was performed as previously described (Talevi and Campanella, 1988).

Composition of solutions used. Ringers (mM): 111.0 NaCl, 2.0 KCl, 1.3 CaCl<sub>2</sub>, 0.8 MgSO<sub>4</sub>; Hepes 25.0, pH 7.6. 10% Ringers (mM): 11.1 NaCl, 0.2 KCl, 0.13 CaCl<sub>2</sub>, 0.08 MgSO<sub>4</sub>; Hepes 2.5, pH 7.6.

## Microscopy

The eggs were observed at various post-fertilization stages under both a Zeiss stereomicroscope and a Leitz Orthomat compound microscope. Upon positioning of the egg, with a lateral view of the dimple and the concavity, plug dissolution sequence was followed. Photographs were made using Ilford Pan F film.

Some eggs were fixed at different times following fertilization. For light microscopy the samples were fixed in Carnoy's fixative, embedded in methyl methacrylate (JB-4 by Polyscience) and sectioned with a Reichert-Jung 2040/Autocut microtome. Four-micron thick sections were stained with PAS. Photographs were made using Ilford Pan F, Kodacolor, Ektachrome films. For electron microscopy the eggs were fixed in glutaraldehyde and embedded in Epon, as previously described (Talevi and Campanella, 1988).

## Sulfhydryl and disulfide group determination, in vitro tests, protein assay

Eggs were washed in 1 mM HCl. Plugs from unfertilized eggs were manually dissected from the rest of the jelly coats. Similarly, plugs of

TABLE 1

## CONCENTRATION OF THIOLS AND DISULFIDE GROUPS IN PLUGS AND $J_3$ FRACTIONS OF UNFERTILIZED AND FERTILIZED EGGS OF *DISCOGLOSSUS*

	Experiment I		Experiment II		Experiment III			
	plug	plug	plug	plug	plug	plug	jelly $J_3$	jelly $J_3$
eggs	u	f	u	f	u	f	u	f
	14		28		25		3	
µg protein/egg	17.1	27.1	10.7	13.6	5.3	4.0	25	15
-SH (nmol/egg)*	0.26	2.60	0.20	2.80	0.30	0.85	<0.13	<0.1
-S-S- (nmol/ egg)*	1.35	0.14	0.80	0.45	0.95	0.18	0.9	0.7
-SH (nmol/mg protein)*	15	94	15	207	60	246	<5	<6
-S-S- (nmol/mg protein)*	79	5	71	33	180	46	43	49

\*Figures refer to data obtained after urea denaturation; u, unfertilized; f, fertilized.

fertilized eggs were manually removed at various stages of liquefaction.  $J_3$  from unfertilized and fertilized eggs were also dissected from the egg and utilized as controls.

About twenty plugs or five  $J_3$  were sonicated with an exponential tip sonicator (Lab Line Instr. Melrose, Il., U.S.A.) in ice for 20 sec at 40 W power, in 1 mM EDTA in order to solubilize the gelatinous matrix at a low pH and to avoid reoxidation of free sulfhydryl groups.

Titration with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman, 1959) was performed either on the sonicated solution as such or after TCA precipitation. The solution added with TCA (11% final concentration) was centrifuged after 15 min in the cold, and both the precipitate and the supernatant were collected. The proteic precipitate was dissolved in water by sonication.

For *in vitro* tests, aliquots of unfertilized eggs were dejellied by the DTT treatment, rinsed in 1/10 Ringer, and immersed in 50  $\mu$ M A23187 for 2 min, then rinsed again. In the next 3-4 min there was contraction of the dimple followed by its disappearance, thus indicating that activation, including primary exocytosis, had occurred (Talevi *et al.*, 1985; Campanella *et al.*, 1986). About 15 min later, the eggs underwent secondary exocytosis, as evidenced by the transient whitening of the regressed dimple region. The activity of the Ringers containing "primary exocytosis" or "secondary exocytosis" products was tested on dissected plugs.

Tests for -SH were performed as follows. Samples (100-500  $\mu$ l) were adjusted to pH 8.5 by addition of diluted NaOH and Tris buffer, pH 8.5 (0.1 M final concentration) DTNB (1 mM in a final volume of 3.0 ml) was added, and absorption at 412 nm was recorded until constant values were reached (about 20 min).

To evaluate the concentration of disulfide groups, neutralized samples were first added with NaBH<sub>4</sub> (1-2 mg) and allowed to react for 30 min at room temperature. The solution was then acidified with 1 M HCl to destroy the reagent excess, brought to pH 8.5 with NaOH and Tris buffer pH 8.5 and added with DTNB as above. The amount of -SH groups was calculated by using the value  $\Sigma = 13,600$  M cm (Ellman, 1959). The amount of -S-S- groups was calculated from the difference between the two sets of data. Determinations were in duplicate.

In some tests the solution, either as such or after TCA precipitation, was heated at 60°C in the presence of 6M urea before being processed further.

Proteins were determined by the Bio-Rad method, using BSA as standard protein.

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