Zinc Uptake and Distribution in *Xenopus laevis* Oocytes and Embryos[†]

K. H. Falchuk,[§] M. Montorzi,[‡] and B. L. Vallee*,[‡]

Center for Biochemical and Biophysical Sciences and Medicine and Department of Medicine at the Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: *Xenopus laevis* vitellogenin contains 2 g-atoms (g-at) of Zn and 3 g-at of Ca/dimer, transports zinc in plasma, and plays a role in its distribution within the oocyte [Montorzi et al. (1994) *Biochem. Biophys. Res. Commun. 200*, 1407−1413; Montorzi et al. (1995) *Biochemistry 34*, 10851−10858]. We here report the dynamics and time course of Zn^{65}-labeled vitellogenin uptake by and distribution within stages II and IV oocytes, the fate of the metal in oocytes as they progress from stages II to VI, as well as in the first two cleavage blastomeres, the blastula, and subsequent stages of the developing embryo and tadpole. Zn^{65} bound to vitellogenin is taken up within less than 30 min by either stage II or IV oocytes incubated under *in vitro* culture conditions whereas free Zn^{65} is not. Once internalized, Zn^{65} remains within the cytosol of stage II, whereas in stage IV oocytes, it is transferred within 4 h of its entry from the cytosol into yolk platelets. Nearly all of the transferred Zn^{65} is found within yolk platelets and their precursors where it is associated with the vitellogenin cleavage product, lipovitellin. Its distribution within the oocyte organelles differs at each stage of oogenesis. In the early stages (III−IV) most of the oocyte zinc is located first in the small endocytosed vesicles and then in multivesicular bodies. When the zinc transfer process is finalized in the late stages of oogenesis (V−VI), >90% of the total oocyte zinc is within yolk platelets while the remainder is in the cytosol. In embryos and tadpoles, the larger of these two pools remain sequestered in yolk platelets and is inaccessible to cytosolic apoproteins throughout the entire period of embryo formation. Its redistribution to the cytosol does not begin until several days after the tadpole has hatched. The smaller pool, on the other hand, is already present in the cytosol and is, therefore, postulated to constitute the sole source of zinc required for embryogenesis.

The ubiquitous occurrence of zinc in biology and its participation in the biochemistry of all living systems require regulation of its transport in plasma, and its uptake by and distribution within cells to ensure that apoproteins are supplied with the metal in the quantities needed and at the time required to carry out their functions (Vallee & Falchuk, 1993). Remarkably these mechanisms are largely unknown at present. The *Xenopus laevis* oocyte/embryo system seems particularly well suited for their identification. Large numbers of oocytes can be obtained at each stage of oogenesis and embryos at different points of development. Established methods for their study under physiological and pathological conditions are well suited to address zinc uptake and distribution. Thus, oocytes take up zinc throughout the entire period of oogenesis until they attain a concentration of 1 mM at stage VI. However, once fertilized, the developing embryo does not receive additional zinc and seemingly contains enough for its entire formation (Nomizu et al., 1993). Therefore, any zinc needed to form holoenzymes from apoproteins synthesized during embryogenesis must be acquired from stores existing within the organism. Curtailment by chelating agents of zinc available from such putative stores has proven teratogenic to embryos (Jörnvall et al., 1994).

Such observations and conclusions have led us to initiate a systematic examination of the mechanism(s) of zinc uptake by and distribution within oocytes, and of the macromolecules into which it is incorporated into and its utilization by embryos and tadpoles. The demonstration that vitellogenin is a zinc metalloprotein containing 2 g-atoms (g-at) of Zn/dimer identifies its central role in both zinc transport in plasma and zinc uptake by cells and accounts for the accumulation of zinc in oocytes (Nomizu et al., 1993; Montorzi et al., 1994). Moreover, once vitellogenin is taken up, it is processed into lipovitellin which also is a zinc metalloprotein and, thereby, participates in zinc distribution within the oocyte (Montorzi et al., 1995).

We have now examined the kinetics of zinc vitellogenin uptake by and its distribution within oocytes once the carrier protein is processed at various stages of oogenesis. Concurrently, the fate of the zinc has been determined in individual early cleavage blastomeres and subsequent embryo and tadpole cells as they progress through the first 40 stages of frog development. Employing Zn^{65} vitellogenin, we demonstrate that the metal is transported into both "previtellogenic" stage II and "vitellogenic" stage IV oocytes as a zinc vitellogenin complex. However, once internalized, the distribution of Zn^{65} differs characteristically; it remains within a cytosolic component of stage II oocytes while it is deposited progressively into yolk platelets of stage IV ones. The compartmentalization of zinc into these two discrete pools continues throughout the remaining stages of oogenesis and terminates when stage VI is reached. Sucrose gradient centrifugation separates the two zinc compartments, revealing that the yolk platelet zinc fraction (pool I) accounts for 90%

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* Author to whom correspondence should be addressed.
[‡] Center for Biochemical and Biophysical Sciences and Medicine.
[§] Department of Medicine.

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of the total, remains unchanged throughout the entire period of embryonic development, and becomes available to any newly formed apomolecule several days after tadpole hatching. The second, smaller fraction (pool II), which contains the remaining 10% of the egg zinc, remains in the cytosol beginning with fertilization. We suggest that it is the zinc that is incorporated into newly synthesized cytosolic apoproteins that renders them functional during embryogenesis.

These results now allow the identification of zinc macromolecule(s) in the cytosolic compartment of oocytes and the examination of this metal's distribution among acceptor apoproteins within this cell space during the first major developmental events encompassed by embryogenesis. Similarly, the fate of yolk platelet zinc can also be examined in terms of the corresponding macromolecules essential for the second vital developmental process: early metamorphosis.

MATERIALS AND METHODS

All plastic and glassware used for storage of buffers and for preparation and analysis of metals were soaked overnight in 3 N HNO_3 and washed extensively with ultrapure water before use to prevent adventitious metal contamination. Buffers and other solutions were treated routinely with Chelex 100 resin to remove metal contaminants (Falchuk et al., 1988).

In Vitro Uptake of Zn^{65} Vitellogenin. Vitellogenin was purified as previously described (Montorzi et al., 1994). Its synthesis was induced by two injections of 4 mg of estrogen (Sigma, St. Louis, MO), each administered seven days apart into the dorsal lymph sac of female *Xenopus laevis* frogs, 6−7 cm long, obtained from Xenopus I (Ann Arbor, MI). One week later, the animals were sacrificed, blood was collected, and serum was obtained by centrifugation. Aliquots of serum were adjusted to contain 35% saturated $(NH_4)_2SO_4$ followed by centrifugation at 50 000*g*. The supernatant was dialyzed against 25 mM Hepes, 1 mM PMSF, 500 μM leupeptin, pH 7.5, and 1-mL aliquots were applied to a Mono-Q column HR 5/5 (Pharmacia, Uppsala, Sweden) equilibrated with 25 mM Hepes, pH 7.5 at 22 °C. The sample was eluted with a linear gradient ranging from 0 to 0.5 M NaCl in 25 mM Hepes, pH 7.5 buffer. Absorbance of the eluate was monitored at 254 nm. Leupeptin was added to the eluate to achieve a final concentration of 1 mM. The zinc content of the purified material was determined by atomic absorption spectrometry (Montorzi et al., 1994).

To label vitellogenin with Zn^{65}, aliquots of the zinc protein were dialyzed overnight against 500 volumes (vol) of 1 mM 1,10-phenanthroline in 25 mM Hepes, 0.5 mM leupeptin, at pH 7.5, 2 °C, and then against the same buffer but without chelating agent for an additional 12-h period. The dialyzed apoprotein sample was mixed with 0.1 vol of 0.5 M MES, pH 6.1, and a 2.5-mL aliquot containing 70 μg protein was added to a water solution containing 6.6 nmol of Zn^{65} (total counts 1 700 000 cpm). After a 1-h incubation, the sample was dialyzed overnight against 400 vol of zinc-free 25 mM Hepes, pH 7.5, 2 °C. The radioactivity associated with the protein, the dialysis bag, and the dialysate were measured with a Searle Model 1185 Gamma Counter System.

Viable oocytes were obtained to study the *in vitro* uptake of free Zn^{65} and Zn^{65} vitellogenin. Frog ovaries were removed, and the fibrous sheath surrounding the ovarian lobes was dissected as much as possible before incubating the tissue with 0.5 mg/mL of collagenase Type II (Worthington) in OR-2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.8) at 22 °C for 4 h (Opresko, 1991). The oocytes were then washed in OR-2 medium but without collagenase; thereafter aliquots of stage II, III, IV, V, and VI oocytes were separated based on their size and morphology (Nomizu et al., 1993). Stage II and IV oocytes were placed in fresh OR-2 medium and left in the cold room overnight. Oocytes that became pale and discolored were discarded. Aliquots of viable stage II or IV oocytes (40−50) were then transferred into dishes containing 20 μg Zn^{65} vitellogenin/mL of OR-2 media. One dish was placed at 4 °C and the others were left at 22 °C. After a 30-min incubation period, the oocytes were washed extensively with fresh OR-2 medium and reincubated with unlabeled vitellogenin at a final concentration 500 μg/mL of OR-2 medium. Oocytes were withdrawn from the dishes after 0.5, 1, 5, 6.5, and 25.5 h later. Zn^{65} was incubated with separate aliquots of both stages II and IV oocytes for 26 h. The total number of counts of free Zn^{65} in the incubation chambers were identical to those used in the experiments with Zn^{65} vitellogenin.

Zinc Distribution in Homogenates of Oocytes at Different Stages of Development. Oocytes incubated with Zn^{65} were examined first. All oocytes were washed × 3 with 0.25 M sucrose, 25 mM Hepes, 500 μM leupeptin, pH 7.5 to remove adherent Zn^{65} or radiolabeled vitellogenin. The oocytes were homogenized, and 1-mL aliquots were layered on sucrose gradients prepared with 40 mM Hepes, pH 7.5, as diluent. Gradients were organized in 12-mL polyallomer tubes with 1-mL layers of densities: 1.27, 1.24, 1.23, 1.22, 1.21, 1.18, 1.16, 1.12, 1.08 g/mL. Samples were centrifuged at 50 000*g* in an SW 40 rotor at 2 °C for 15−20 h (Jared et al., 1973; Wall & Meleka, 1985; Wall & Patel, 1987). After centrifugation, fractions of approximately 500 μL were collected manually from the top and transferred into polypropylene tubes by means of a peristaltic pump and a fraction collector used in the drop counting mode. The separated fractions were taken for analysis of radioactive zinc and protein.

Separate aliquots of stage II, III, IV, V, and VI oocytes not exposed to Zn^{65} also were homogenized gently in 2−3 vol of buffer containing 40 mM Hepes, 0.25 M sucrose, 1 mM PMSF, pH 7.5, 2 °C. One milliliter of each homogenate was layered onto a prepared sucrose gradient, centrifuged, and collected as described above. The protein content of each homogenate fraction separated on the sucrose gradients was determined with a 1-μL aliquot by the Bio-Rad total protein assay. Spectrophotometric analyses were performed with a Beckman DU 7500 spectrophotometer. The zinc content of each fraction was determined also after boiling aliquots for 60 s in 2 mL of metal-free 15 N HNO_3. Each of the sucrose gradient fractions of stages V and VI oocyte homogenates was examined individually by light microscopy, and the numbers of light and heavy yolk platelets present were determined by counting with a hemocytometer or Coulter Counter.

Zinc Content of Individual Early Cleavage Blastomeres. Frogs, 6−7 cm long, were used in all *in vitro* fertilization experiments. Ovulation was induced by an initial injection of 50 IU of human chorionic gonadotropin into the dorsal lymph sac of female frogs, followed by a second injection of 500 IU 48 h later. Ovulation normally occurred 12−14 h after the second injection. Frog testes were carefully dissected and placed in a solution of 80% Steinberg's

solution. The latter was prepared by diluting a stock solution of 100% Steinberg's standard buffer composed of 58 mM NaCl, 0.67 mM KCl, 0.4 mM $MgSO_4$, 0.34 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.5. The testes were macerated, and sperm viability was determined by visual inspection with a light microscope. Ovulated mature eggs were fertilized by placing them directly into the sperm suspension. Embryos were allowed to remain in stage 1 for 15−20 min before the 80% Steinberg solution was diluted with metal-free water to a final concentration of 20%.

The gelatinous envelopes of 20−30 stage 1 embryos were removed by treatment with 0.16 M NaOH, 2% L-cysteine for 2−3 min followed by extensive washing with 20% Steinberg solution. After the washing step, embryos were placed in fresh 20% Steinberg solution at 22 °C. Embryos were removed from their incubation chamber following the first and second cleavages, stages 2 and 3 of development, respectively. Stage VI oocytes as well as stages 1, 2, and 3 embryos were fixed in 5% glutaraldehyde, 5% ficoll (400 kDa), 25 mM Hepes, pH 7.5 for 60 min. Individual blastomeres were obtained after removing the fertilization membranes of fixed embryos. The stage 2 embryo blastomeres were separated along their cleavage planes by using ultrafine forceps (Dumont). The same technique was used to separate the two ventral and two dorsal cells from stage 3 embryos. The dorsal sectors were distinguished from the ventral ones by their larger size and lighter pigment distribution. Each separated blastomere from stage 2 and the two blastomeres corresponding to the dorsal and ventral regions from stage 3 embryos were transferred with a Pasteur pipette into a plastic petri dish containing 20 mL of ultrapure water (Milli-Q system, Millipore Corporation, Bedford, MA). Glutaraldehyde-fixed oocytes, embryos, and blastomeres were prepared for zinc analysis by washing 3 times with metal-free water, using previously described techniques (Nomizu et al., 1993). The washed eggs, embryos, and blastomeres were then placed in individual Eppendorf tubes, where any remaining water was removed with a micropipette. Each sample was digested in 200 μL of metal-free HNO_3 by boiling for 4 min. Unfixed, unfertilized oocytes and embryos were similarly washed and digested in order to ensure that the fixation step did not contaminate or otherwise alter the zinc content of eggs or embryos.

Zinc Distribution in Cytosol and Yolk Platelets of Developing Embryos and Tadpoles. Sixty embryos and tadpoles at various stages of development (from stages 1 to 40) were homogenized in 1.1 mL of 40 mM Hepes, 0.25 M sucrose, 1 mM PMSF, pH 7.5 at 2 °C. One milliliter of each homogenate was layered on top of a sucrose gradient organized with 500 μL and 4 mL of sucrose at densities of 1.25 and 1.18 g/mL, respectively. The gradients were centrifuged at 50000*g* in an SW 40 rotor at 2 °C for 20 h, and the resultant fractions separated and analyzed for zinc.

Zinc Analyses. The samples were analyzed for zinc by atomic absorption spectrometry with a Perkin Elmer 4100 ZL instrument equipped with a transverse heated graphite furnace. Since matrix modifier was not added, the ashing (pyrolysis) and atomizing temperatures, ramp and hold speeds, and sample volumes injected were optimized to operate at maximum signals. The furnace was preheated at 80 °C to reduce drying time. Standards and samples were read twice and accepted when the relative difference between the two readings was less than 10% at a concentration higher than 2 ng/mL. Only standard curves with a correlation coefficient >0.99 were used in zinc analyses. Samples were diluted with ultrapure water containing metal-free 0.2% HNO_3 to adjust their zinc content to within the range of the standard curves.

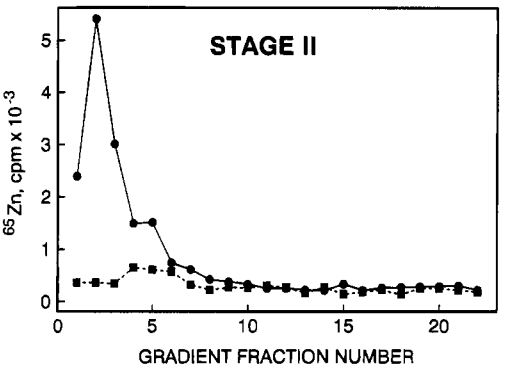

FIGURE 1: Stage II oocyte Zn 65 uptake. Stage II oocytes were incubated with either Zn^{65} vitellogenin (●) or free Zn^{65} (■). Following a 30-min incubation, the oocytes were homogenized and the homogenate was centrifuged in a sucrose gradient. Zn^{65} enters the oocyte when bound to vitellogenin, and remains in a cytosolic region. No change in its distributions within the gradient is observed after 26 h of incubation (not shown). Free Zn^{65} does not enter the oocyte.

Gel Electrophoresis. The molecular weights of protein subunits from vesicles and yolk platelets were estimated by SDS polyacrylamide gel electrophoresis (SDS−PAGE) using a 6% stacking gel and a 9% resolving gel. Gels were run at 40 mA and stained with 0.1% Coomassie blue, 50% methanol (v/v), 10% acetic acid (v/v), and 0.1 M $AlCl_3$.

RESULTS

Zn^{65} Transport into and Distribution within Stage II and IV Oocytes. Purified plasma vitellogenin contains 2 g-at of zinc/440 kD, as previously described (Montorzi et al., 1994, 1995). Dialysis against 1 mM 1,10-phenanthroline removes all zinc from the protein. Incubation of the apoprotein with Zn^{65} reconstitutes the holoprotein. Stages II and IV oocytes incubated in OR-2 medium remain viable for more than 24 h. In control studies, neither stage II nor IV oocytes take up free Zn^{65} ions during an incubation period of 26 h. In contrast, within 30 min of incubation, Zn^{65}-bound vitellogenin enters both stage II and IV oocytes (Figures 1, 2).

The fate of internalized ^{65}Zn vitellogenin has been examined in the light of the known processing of the protein. Once taken up by oocytes, vitellogenin is processed within small endocytosed vesicles, multivesicular bodies, and yolk platelets. These three types of organelles have been separated based on their different densities in a sucrose gradient and identified further by either visualization by light microscopy and/or the presence of the characteristic phospholipoproteins which they are known to contain (Wallace et al., 1972; Jared et al., 1973; Wallace, 1978; Wahli et al., 1981; Wall & Meleka, 1985. Wall & Patel, 1987).

Yolk platelets sediment to the regions of gradient with the highest sucrose density (between 1.20 and 1.24 g/mL). They are round to oblong structures large enough to be visualized directly and counted by light microscopy. The yolk platelets are distributed bimodally within the gradient (Figure 3). The lighter platelets are at one extreme, at a density of about 1.21 g/mL, and the heavier ones at the other extreme, at 1.23 g/mL (Wall & Meleka, 1985; Wall & Patel, 1987). These light and heavy yolk platelets vary in number

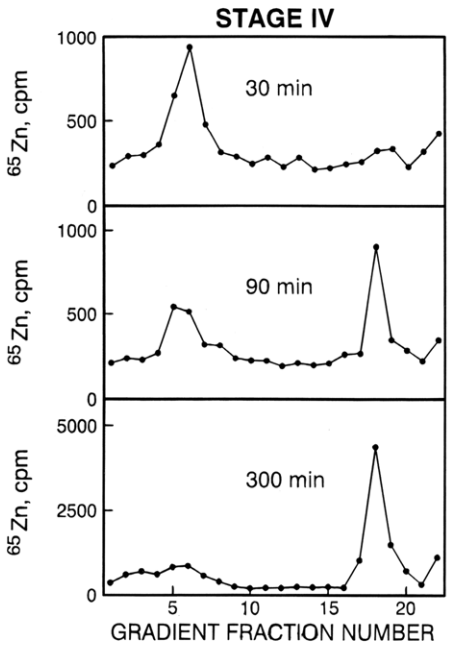

FIGURE 2: Stage IV Zn^{65} uptake. Stage IV oocytes were incubated as described in Figure 1. Within 30 min, Zn^{65} vitellogenin is found within multivesicular bodies and is progressively transferred into yolk platelets, so that by 300 min nearly all of the Zn^{65} is within these organelles. Free Zn^{65} does not enter stage IV oocytes.

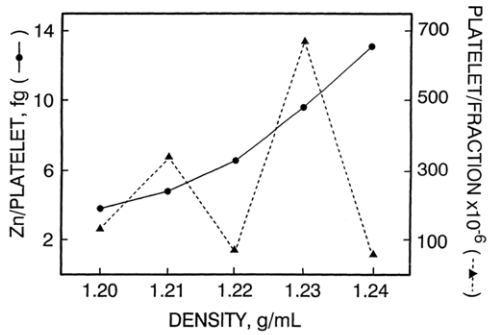

FIGURE 3: Zinc content/platelet. There is a progressive increase in zinc contact/platelet (●) as the organelles increase in density and mature from light to heavy platelets (▲).

depending on the degree of oocyte development. Thus, stage V and early stage VI oocytes contain both light and heavy yolk platelets. Late stage VI oocytes, on the other hand, contain almost exclusively heavy platelets. The proteins found in the yolk platelet-rich regions of the gradient are comprised of three predominant species that separate on SDS−PAGE into a broad band migrating at about 110 kDa and two bands at 35 to 30 kDa (Figure 4, lane 5). These have been identified as the vitellogenin cleavage products, lipovitellin subunits 1 and 2 and phosvitin, on the basis of their amino acid compositions and N-terminal sequences, (see below).

The less dense multivesicular bodies are too small to be visualized by light microscopy. However, their presence in sucrose gradient fractions (density between 1.14 and 1.16 g/mL) has been established previously using electron microscopic techniques and by the observation that they, too, contain the vitellogenin cleavage products lipovitellin and phosvitin (Wallace et al., 1972; Jared et al., 1973; Wallace, 1978; Wahli et al., 1981; Wall & Meleka, 1985, Wall & Patel, 1987). In the present studies, lipovitellin and phosvitin are the proteins found in the region of the sucrose gradient expected to contain multivesicular bodies (Figure 4, lane 4).

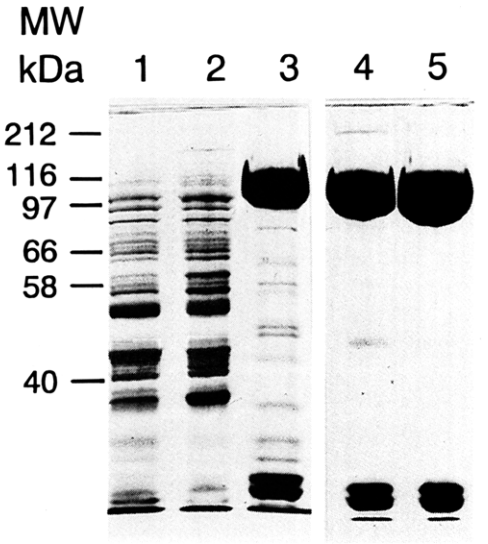

FIGURE 4: Distribution of proteins within stage IV oocyte homogenate fractions separated by SDS−PAGE. The types, amounts, and sizes of proteins found in cytosolic fractions (lanes 1 and 2) differ from those of endosomes (lane 3), multivesicular bodies (lane 4), and yolk platelets (lane 5). The yolk platelet proteins are lipovitellin and phosvitin.

Small endocytosed vesicles have been shown previously to be present in the sucrose gradient region that sediments between 1.08 and 1.12 g/mL (Wall & Meleka, 1985; Wall & Patel, 1987). Those fractions contain multiple bands with mobilities that correspond to polypeptides ranging from at least 120 to 20 kDa (Figure 4, lanes 1 and 2), as previously reported (Denis & le Maire, 1972; Hanas et al., 1983; Miller et al., 1985).

Sucrose gradient analyses of homogenates from oocytes preincubated with Zn^{65} vitellogenin reveal that the presence of Zn^{65} in intraoocyte compartments depends on the stage of oogenesis. Furthermore, any progression of internalized Zn^{65} from one to another of these compartments, corresponding to cytosol and all three types of organelles—small endocytosed vesicles, multivesicular bodies, and yolk platelets—is readily determined by pulse-chase experiments. In stage II oocytes, all of the Zn^{65} is localized entirely to the cytosolic fluid overlaying the sucrose gradient and the adjacent 1.08−1.12 g/mL sucrose density layers (Figure 1). The Zn^{65} remains in those fractions throughout the entire experimental period of 26 h. In contrast, the distribution of Zn^{65} within the more developed stage IV oocytes changes in the first 300 min of incubation (Figure 2). Thus, after 30 min, the major peak of Zn^{65} is found in the 1.10−1.12 g/mL gradient fractions, the region of multivesicular bodies. After 90 min, a second zinc peak appears in the 1.20−1.24 g/mL region, where light and heavy yolk platelets are found. In the course of the next 300 min, >90% of the Zn^{65} localizes to the 1.20−1.24 g/mL (yolk platelet) regions of the gradient (Figures 2−4). Once achieved, this final distribution of zinc remains unchanged for the next 20 h of the experimental period.

Zinc Distribution in Stage II−VI Oocyte Homogenates. Transfer of zinc from a cytosolic compartment of yolk platelets is a characteristic of oocyte development. The zinc content within oocytes at different stages from II to VI has been determined by direct atomic absorption spectrometric analysis of fractions separated by sucrose gradient centrifugation. Most of the zinc in stage II oocytes is in the compartments composed of cytosol and small vesicular

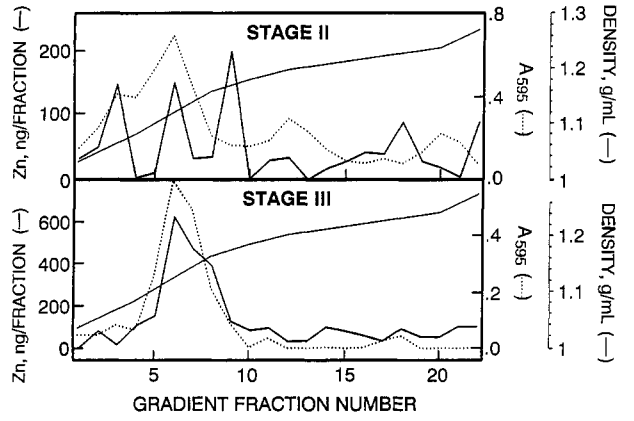

FIGURE 5: Distribution of zinc within homogenate fractions of stages II and III oocytes separated by sucrose gradient centrifugation.

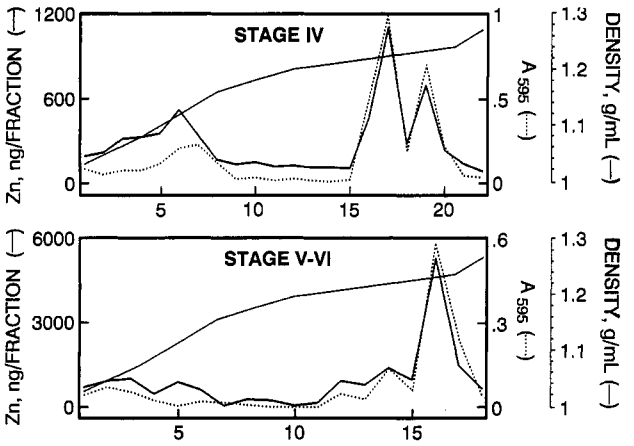

FIGURE 6: Distribution of zinc within homogenate fraction of stages IV, V, and VI oocytes separated by sucrose gradient centrifugation.

bodies. Within these latter two intracellular spaces the zinc is distributed in three distinct peaks (Figure 5). The rest is apportioned over a number of gradient fractions with a small peak in the 1.20−1.24 g/mL region. In stage III homogenates, the zinc peak in the 1.14−1.16 g/mL regions has increased by about 4-fold over that in stage II and predominates as compared to all other zinc-containing fractions (Figure 5). In stage IV an even larger (over 2-fold) biphasic zinc fraction appears in the denser (1.20−1.24 g/mL) regions of the gradient, with the lighter of the two predominating (Figure 6). In stage V−VI oocytes, it is the denser of the biphasic peak that now becomes the major one. Thus, as oocytes develop, a greater fraction of their zinc is found in yolk platelets until by stage VI two distinct pools are established. In the fully mature egg, approximately 10% of the zinc is within the cytosol and the 1.08−1.12 g/mL region, while the remaining 90% is concentrated in the 1.20−1.24 g/mL region. This distribution of zinc into a yolk platelet (hereafter referred to as pool I) compartment and a broad cytosolic one (hereafter referred to as pool II) is ismilar to that of Zn^{65} 300 min after internalization of Zn^{65} vitellogenin by stage IV oocytes (Figures 1, 2, and 6).

The zinc fractions in the pool I region contain large numbers of yolk platelets readily identified by microscopy. Repeated washing and centrifugation of the pool I followed by zinc analysis of supernatants and organelles reveals that the metal is contained entirely within the platelets. Moreover, their zinc content increases as a function of their density (Figure 3). The lightest yolk platelets (located in the 1.20 g/mL fraction) contain a zinc/organelle ratio nearly a fourth of that found in the heaviest ones (1.24 g/mL). The zinc within the platelets is bound to the yolk protein component, lipovitellin (Montorzi et al., 1995).

Table 1: Stage VI Oocyte and Embryo Zinc Content

sample	control Zn (ng)	glutaraldehyde fixed Zn (ng)
oocyte, stage VI	66.8 ± 5	66.9 ± 6
embryo, stage 1	77.7 ± 4	69.5 ± 6
embryo, stage 2	67.0 ± 3	69.0 ± 5
embryo, stage 3	69.6 ± 7	70.3 ± 2

Table 2: Early Cleavage Blastomere Zinc Content

cleavage	blastomere	Zn (ng)
First	1	36
	2	38
Second	ventral duplet	32
	dorsal duplet	43

Zinc Distribution in Individual Early Cleavage Blastomeres and Embryo Intracellular Compartments. The identification of the existence of two zinc pools in mature stage VI oocytes called for an analysis of zinc in these compartments throughout embryonic development. A quantitative analysis of total zinc in each of the early blastomeres generated by the first two cleavages as well as that in each of the two compartments of embryos and tadpole cells in the first 40 stages of development was carried out first.

The amount of zinc in stage 1−3 embryos is the same as that in stage VI eggs, and glutaraldehyde fixation does not alter their zinc content (Table 1). The first cleavage in the frog embryo occurs about 1 h after fertilization and results in two blastomeres of equal size. The second cleavage takes place along the ventral dorsal plane and creates the larger dorsal and the smaller ventral sectors. These have been identified on the basis of their gross morphologies. Glutaraldehyde fixation allows the embryos and blastomeres to be manipulated while preserving their physical integrity. The fertilization membrane is easily removed by careful dissection without disrupting the embryos. The two blastomeres of stage 2 and the dorsal and ventral duplets of stage 3 embryos can be separated manually without damage. Furthermore, the blastomeres are not visibly altered by the extensive washing in distilled water intended to remove any zinc contamination resulting from membrane dissection and subsequent manipulations. Following the first cleavage, zinc is distributed equally between the resulting two blastomeres. However, after the second cleavage, the zinc content of the two larger dorsal blastomeres is about 30% higher than that of the ventral ones (Table 2). The sum of the amounts of zinc found in each blastomere equals that of the intact embryo. Hence, zinc is not lost from or added to the fixed blastomeres during fixation or preparation for metal analysis.

Within the intracellular space of the blastomeres, zinc is apportioned into the same two asymmetric pools described above for stage VI eggs. The larger of the two contains >90% of the zinc and is readily separable from the smaller one with about 10% (Figure 7), corresponding to those identified in stage VI oocytes (Figure 6). The two pools are present at all stages of embryonic development. The amount of zinc in each pool remains constant during the entire cleavage and gastrulation periods and until the embryo has formed a tadpole which is hatched at about 25−30 h

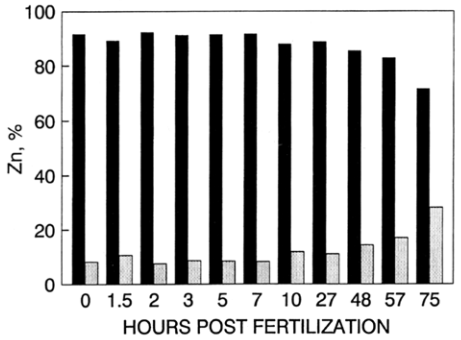

FIGURE 7: Changes in zinc content of cytosol (stippled bars) and yolk platelet (solid bars) fractions in embryos and tadpoles at different stages of development. Yolk platelet zinc content of remains unchanged until after hatching (about 30−35 h post-fertilization) after which it is transferred to the cytosol.

post fertilization. Between that event and during the next 50 h, as tadpoles progress beyond stage 40, about 50% of the zinc associated with platelets (pool II) is released and transferred to the cytosolic pool I (Figure 7).

DISCUSSION

Throughout the 3-year period required for the complete maturation of oocytes, they take up and store maternally derived zinc and other nutrients as well as nucleic acid and protein precursors (Grant, 1953; Dumont, 1972; Gilbert, 1985; Gurdon & Wakefield, 1986; Hansen & Riebessel, 1991; Nomizu et al., 1993). Once the egg is fertilized, embryos employ these nutrients and constituents to synthesize large numbers of proteins some of which must incorporate zinc in order to become functional. We have reported that during the interval it takes embryos to progress from a single cell organism into a multicellular tadpole, they do not acquire any zinc from their external environment. Therefore, the only zinc available to be redistributed within the embryo to those newly synthesized apoproteins is that which accumulates during oogenesis and maturation.

We continue here to delinate the biochemistry underlying zinc uptake, distribution and utilization in frog oocytes and embryos (Nomizu et al., 1993; Jornvall et al., 1984; Montorzi et al., 1994, 1995). As a first step, we utilized stage II and IV oocytes to determine whether zinc enters them as an ion or is carried in bound to a protein. Oocytes incubated in media with free Zn^{65} ions do not incorporate the metal at all. The selection of a candidate protein that could be involved as a zinc transporter was made on the basis of previous data which demonstrated that oocyte zinc content increases progressively as they develop and that the major increase occurs during stages III−VI. The coincidence of zinc uptake with the entry of vitellogenin suggested that the two events are correlated and pointed to the design of experiments to confirm the postulate (Nomizu et al., 1993). As a prelude to the examination of the relationship between vitellogenin and oocyte zinc uptake, we first purified the protein from estrogen-stimulated female frog plasma as previously described, and demonstrated that it contains 2 g-at of zinc/dimer (Montorzi et al., 1994, 1995). We have now studied its role as a zinc transporter into the oocyte. The vitellogenin preparations used for the *in vitro* uptake studies were homogeneous and not contaminated with albumin or other plasma proteins that might bind or carry zinc into the oocyte and complicate the interpretation of the results. Homogeneous vitellogenin was rendered zinc free by dialysis against 1,10-phenanthroline under conditions detailed elsewhere (Montorzi et al., 1995). Direct analysis of the apoprotein by atomic absorption spectrometry confirmed the absence of zinc. Reconstitution with Zn^{65} was carried out at pH 6.1, where it has been demonstrated to be effective. Special care was taken to ensure that the oocytes selected for the *in vitro* zinc uptake studies were viable.

Operationally, the entire oogenesis process has been subdivided into "previtellogenic" and "vitellogenic" phases. In the latter, corresponding to stages III−VI, vitellogenin uptake through receptor-mediated endocytosis is a predominant event in the development of the oocyte (Yusko & Roth, 1976; Stifani et al., 1990). The peak uptake rate occurs in stage IV. Therefore, this stage was utilized. As shown, vitellogenin functions as a zinc carrier into stage IV oocytes (Figure 2). The results demonstrate that Zn^{65} enters the oocyte only when associated with vitellogenin; in these studies oocytes would not have taken up any free Zn^{65} ions that might have been released from vitellogenin into the incubation medium. In stages I and II, the previtellogenic phases, the oocyte has been thought to be entirely devoid of vitellogenin (Gilbert, 1985; Hansen & Riebessel, 1991). Since the zinc content increases as oocytes progress from stage I to II, it became necessary to determine whether zinc vitellogenin would serve the same function of bringing the metal into those oocytes or if another protein needed to be identified for the same purpose. In fact, zinc vitellogenin does enter stage II oocytes under the same experimental conditions used with stage IV ones (Figures 1, 2). Perhaps the "previtellogenic" and "vitellogenic" terminology reflects differences that may be quantitative rather than qualitative.

The finding that vitellogenin mediates the entry of zinc into both "previtellogenic" and "vitellogenic" oocytes must be viewed in the context of knowledge regarding vitellogenin uptake and processing. These events have been examined in great detail and can now be used to eluciate the metabolic fate of zinc in both the oocyte and the embryo. In the stage III−VI oocytes, vitellogenin is processed within intracellular organelles of increasing size and density (Hansen & Riebessel, 1991). Vitellogenin binds to receptors which accumulate in coated pits of the oocyte membrane (Yusko & Roth, 1976; Stifani et al., 1990). The ligand−receptor complex is internalized into vesicles that fuse with one another as well as with lysozomes within the cytosol to form endosomes and multivesicular bodies. Vitellogenin then dissociates from its receptor and is cleaved into its major products, lipovitellin and phosvitin. The cleavage appears to be a necessary step for the further fusion of multivesicular bodies to form first light and then heavy yolk platelets that contain condensed and crystalized lipovitellin and phosvitin complexes (Wallace & Jared, 1968; Wallace & Opresko, 1983; Opresko & Karpf, 1987). As would be expected from their origin from the multivesicular bodies, the platelets contain the same major proteins but in much larger amounts per organelle where they are stored in a crystalline form (Wallace et al., 1972; Jared et al., 1973; Wallace, 1978; Wahli et al., 1981). Both lipovitellin and phosvitin have been extensively studied and are well characterized in terms of their solubility properties, electrophoretic mobilities, amino acid composition and sequences, high phosphoserine and lipid contents, etc. (Begink & Wallace, 1974; Wiley & Wallace, 1978; 1981; Germond et al., 1983; Gerber-Huber et al., 1987; Banaszak et al., 1991). We report elsewhere that lipovitelin retains the zinc after vitellogenin cleavage in *X. laevis* oocytes (Montorzi et al., 1995).

The above information allows a number of conclusions regarding the effect of vitellogenin processing on zinc distribution within the oocyte. In both stage II and IV oocytes, vitellogenin is taken up within minutes of incubation but is processed differently. In stage IV oocytes, the changes in sedimentation properties of the Zn^{65} internalized bound to vitellogenin suggest that the zinc remains associated with the protein as it undergoes processing. This is evidenced by the shift in the peak of radiolabeled zinc from regions of the sucrose gradient that correspond first to cytosol, then to multivesicular bodies and light platelets and finally to heavy platelets (Figures 2−4). This same shift is noted when zinc is analyzed by atomic absorption at different stages of oocyte development (Figures 5 and 6). As expected, the zinc content/platelet increases as the organelle evolves from light to heavy (Figure 3) and the zinc lipovitellin content increases (Cook, 1961, 1968; Jared et al., 1973; Opresko et al., 1980a; Wiley & Wallace, 1981; Butler, 1983; Griffin et al., 1984; Griffin, 1985; Wall & Meleka, 1987).

In contrast to stage IV oocytes, the Zn^{65} vitellogenin that enters stage II oocytes remains within a cellular compartment that localizes in the upper region of the sucrose gradient (Figure 1). One possible explanation is that in the stage II oocytes zinc vitellogenin is not processed and, therefore, not transferred into yolk platelets. The fusion of endosomes containing endocytosed receptor−vitellogenin complexes into multivesicular bodies where cleavage of the protein occurs is an early step in vitellogenin processing. It is unknown at present whether stage II oocytes take up vitellogenin through receptor-mediated endocytosis, as in stage IV, or through an alternate pathway, as shown for other proteins, e.g., albumin, that enter oocytes when added to the incubation media (Opresko et al., 1980a,b; Wall & Patel, 1987; Opresko, 1991). If the latter is the case, then vesicular bodies containing internalized vitellogenin−receptor complex would not be formed, a prerequisite for cleavage of the protein. Failure to process vitellogenin might account for its presence in the cytosolic compartment (Figure 1). Alternatively, Zn^{65} could persist in the cytosol if it is released from vitellogenin following entry into stage II oocytes and gets transferred to other cytosolic molecules. Such a mechanism would be analogous to that which operates for iron between the plasma protein transferrin and intracellular ferritin (Theil, 1987). A number of *X. laevis* zinc proteins could be potential acceptor candidates. The 7S and 42S ribonucleoprotein particles are known to be distributed in the pertinent sucrose gradient region (Denis & le Maire, 1972) where the zinc is located in stage II oocytes. Both of these particles contain zinc, and at least one of the proteins associated with the 7S particle, TFIIIA, has been well characterized as a zinc complex (Hanas et al., 1983; Miller et al., 1985). TFIIIA is synthesized mainly during stage I and II, and zinc must be available in the cytosol at that time for full loading of the apoprotein. The zinc protein(s) involved must be among the polypeptides shown in Figure 4, most of which clearly differ from the ones typically found in multivesicular bodies or yolk platelets.

The recognition that in the oocyte zinc is distributed into two compartments called for an examination of these two pools in embryos and their individual blastomeres. The zinc content of blastomeres has not been reported previously, owing in large part to technical difficulties encountered when manipulating and isolating biological cells and tissues and in the preparative methods required to obviate zinc contamination (Falchuk et al., 1988; Nomizu et al., 1993). Thus, standard methods used to isolate frog blastomeres require that the embryos are placed in hypertonic solution (e.g., 5% ficoll) to extract water from the space between the fertilization membrane and the blastomere so that the membrane itself can be removed. Subsequently, the embryo is placed in calcium- and magnesium-free buffers to separate the blastomeres. Isolated blastomeres devoid of their fertilization membrane are fragile and are easily damaged by manipulation in preparation for metal analysis (Keller, 1991; Nomizu et al., 1993). Therefore, we devised a different approach to allow removal of the membrane, separation of blastomeres, and washing of the cells. We first fixed the embryos in glutaraldehyde. This procedure is advantageous since once the blastomeres are fixed, their fertilization membranes can be readily removed and they can be manipulated without difficulty. Finally, fixed blastomeres can be washed in metal free water without being destroyed. The fixation and washing procedures neither contaminate the samples with nor remove any zinc already present, as demonstrated by metal analysis of fixed and unfixed oocytes and embryos (Table 1). The total amount of zinc found in the two blastomeres of stage 2 embryos or in the four blastomeres of stage 3 embryos is about equal to that of the corresponding intact embryos at the same stages (Table 2).

Quantitative analysis of the zinc in individual blastomeres of stage 1−3 embryos and in their homogenates during these and the subsequent stages of embryonic and tadpole development provides information on its distribution in the embryo and its utilization during these periods. One of the earliest events in frog embryo formation is the establishment of dorsal and ventral segments initiated by the second cleavage. This results in an asymmetric distribution of zinc, with the larger part of it in the dorsal segment which forms most of the major organs (Table 2). Within the cytosol and organelles of the blastomeres themselves, the zinc is distributed into two pools that are also quantitatively asymmetric. About 90% of the total is in yolk platelets, where it remains sequestered for the most of embryonic development (Figure 7). The remaining 10% is cytosolic and would appear to be available for distribution to newly synthesized apoproteins. This fraction must be accessible for use in zinc-dependent processes required for embryogenesis. The total quantity of zinc in the cytosolic pool corresponds to about 1 nanogram per oocyte. Although a minor percentage of the total (Nomizu et al., 1993), this is still a significant amount in absolute terms. Differentiated cells in other systems contain from 1 to 10 picograms of zinc (Falchuk et al., 1975; Vallee & Falchuk, 1993). The total number of cells generated during the 10-h cleavage period of *X. laevis* is about 4000−5000 (Gilbert, 1985). All of the zinc required for these cells can be supplied from this pool.

The present findings are consistent with the paradigm that the development process is one of the major biological loci of function for zinc (Vallee & Falchuk, 1993). Thus, the two zinc pools would seem to be created to ensure that sufficient amounts of the metal are available to complete the two major developmental events of embryogenesis and perhaps early metamorphosis independent of the external zinc content of the surrounding environment. The amounts of metal and its distribution within the egg, therefore, would seem to match future utilization. Most of the zinc-dependent metabolic needs for rapid embryogenesis could be met by the readily available cytosolic fraction, whereas that for the

early post-hatching period could use the larger yolk platelet one. This conclusion is also consistent with present understanding of the role for yolk platelet proteins. They represent about 80% of the egg protein and take nearly 2 years to accumulate, process, and store. Their use appears to be reserved for the second, major developmental event leading to the formation of adult frogs which requires dramatic changes in organs, tissues, cells, proteins, and other constituents of the tadpole (Gilbert, 1985). The size of the organism and the greater number of cells involved would call for the larger amounts of zinc present in the platelets.

The mechanism(s) of zinc transfer between zinc proteins in each developmental stage as well as their identities is (are) unknown. The molecules involved share the characteristic that they are all synthesized as apoproteins and need to acquire zinc to be activated and perform functions critical to embryogenesis and metamorphosis. Among the potential zinc acceptor proteins that could be candidates are the superfamily of steroid hormone receptors which exhibit characteristic zinc binding structures denominated "zinc fingers, twists, and clusters" (Vallee & Auld, 1993). Other candidates are proteins whose synthesis is coordinated to match particular developmental needs including LIM proteins and any zinc-containing homeobox domains required for organ formation (Vallee & Falchuk, 1993).

An understanding of the zinc storage and distribution process in the frog embryo should facilitate definition of a corresponding system in mammalian embryonic cells, if it exists. Thus, amphibians and mammals undergo many similar embryogenic events and, hence, may share analogous biochemical mechanisms to achieve common zinc-dependent developmental processes. The zinc fraction in the mammalian embryo that corresponds to that of the cytosolic frog pool II would seem to be the one available for embryo development in the higher organisms since there is no yolk platelet fraction in placental mammals.

Furthermore, the delineation of a distribution system for zinc in the embryo might serve to identify one in nonembryonic cells. There is sparse factual information regarding zinc uptake by the intestine, transport in plasma, and cellular uptake in nonembryonic vertebrate organisms (Vallee & Falchuk, 1993). There is even less evidence that zinc is stored in any cellular compartment as part of a system that can accommodate changes in requirement. There is no information on how zinc might be distributed to apoproteins to make them functional once the metal is in cells. Metallothionein has been proposed to be involved in this process either directly (Kägi & Schäffer, 1988) or through the interaction with glutathione (Maret, 1994), but this has not been confirmed by direct experiment in cell systems. It is now possible, therefore, to search for systems in nonembryonic cells that are analogous to the vitellogenin molecule, its cleavage products, and others yet to be found in the egg and/or embryo from the pool II fraction and to examine their participation in the storage and distribution of zinc.

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