An Unusual Lysosome Compartment Involved in Vitellogenin Endocytosis by *Xenopus* Oocytes

DORIS A. WALL and IMAN MELEKA
Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021

ABSTRACT   We have investigated the lysosomal compartment of *Xenopus* oocytes to determine the possible role of this organelle in the endocytic pathway of the yolk protein precursor, vitellogenin. Oocytes have lysosome-like organelles of unusual enzymatic composition at all stages of their development, and the amount of hydrolase activity increases steadily throughout oogenesis. These unusual lysosomes appear to be located primarily in a peripheral zone of oocyte cytoplasm. At least two distinct populations of lysosomal organelles can be identified after sucrose density gradient fractionation of vitellogenic oocytes. Most enzyme activity resides in a compartment of large size and high density that appears to be a subpopulation of yolk platelets that are less dense than most platelets within the cell. The appearance of this high density peak of lysosomal enzyme activity coincides with the time of onset of vitellogenin endocytosis during oocyte development. The data suggest that endocytic vesicles that contain vitellogenin fuse with modified lysosomes shortly after their internalization by the oocyte. Pulse-chase experiments with radiolabeled vitellogenin suggest that the ligand passes through the low density platelet compartment en route to the heavy platelets. The accumulation of yolk proteins apparently results from a failure of these molecules to undergo complete digestion after their entry into an unusual lysosomal compartment. The yolk platelets that these proteins finally enter for prolonged storage appear to be a postlysosomal organelle.

The oocytes of most nonmammalian vertebrates owe their large size primarily to the receptor-mediated endocytosis of yolk protein precursors made by the liver or analogous organs. One of the best-studied examples of this process occurs in the oocyte of the South African frog, *Xenopus laevis*, in which yolk comprises at least 80% of the total protein of a fully grown oocyte (4). In this species, the yolk protein precursor, vitellogenin (VTG)[1], is synthesized by the estradiol-stimulated liver, released into the blood stream, and selectively sequestered by vitellogenic oocytes, which appear to have a plasma membrane receptor for this protein. After internalization, VTG is cleaved into several smaller fragments, including the highly phosphorylated protein phosvitin, and two relatively hydrophobic lipovitellins (27). In this form, the proteins are stored for prolonged periods as lipoprotein crystals within membrane-bound yolk platelets. Yolk proteins do not undergo further proteolysis until much later during embryonic development, when they are degraded to amino acids that serve as a food supply for the embryo (27, 30). The mechanism by which the final catabolism of yolk is prevented in the

oocyte and later initiated in the embryo is currently unknown.

Since most molecules entering cells by receptor-mediated endocytosis are degraded after fusion of endosomes that contain the ligand with lysosomes (24), the latter organelle is probably involved in the VTG pathway. Nevertheless, little information is available regarding lysosomes in the *Xenopus* oocyte, and the data that exist are contradictory. In some reports, examination of vitellogenic oocytes has failed to reveal typical lysosomes, either biochemically or morphologically (7, 28, 30). In others, analysis of mature, ovulated oocytes has revealed low levels of acid phosphatase (6, 23) and substantial levels of β-*N*-acetylglucosaminidase (NAG) and protease activity (6). Lysosomal enzymes did not appear to be associated with yolk platelets in either study. Ultrastructural examination of developing oocytes has been performed by a number of laboratories (1, 8, 32), with multivesicular bodies (MVBs) and/or dense bodies described in some reports but not mentioned in others.

Possible explanations for the events that occur after VTG endocytosis include the following: (*a*) Oocytes may have "typical" lysosomes either before or after the vitellogenic phase during which they accumulate yolk proteins, but production of these lysosomes ceases during VTG accumulation

---

[1] *Abbreviations used in this paper:* MVB, multivesicular body; NAG, β-*N*-acetylglucosaminidase; VTG, vitellogenin.

(3, 30). (b) Endosomes that contain internalized VTG fail to fuse with lysosomes, as observed in the entry of some pathological organisms into cells (5). (c) Oocytes have lysosomes at all stages, but the complement of enzymes present may not include those needed to degrade VTG. Alternatively, the absence of degradation could result from inadequacy of the lysosomal environment such as failure or reversal of the normal acidification of endosomes and/or lysosomes that occurs on the endocytic pathway (16, 25). Any such deficiency would presumably be corrected during embryogenesis, when yolk degradation finally occurs.

The work reported in this paper demonstrates that oocytes have modified lysosomes of unusual enzymatic composition throughout their development. Vitellogenic oocytes have at least two distinct populations of lysosomes, and the major one appears to coincide with a yolk platelet fraction that is a minor subpopulation of the total platelets within the cell. Yolk accumulation appears to result from the failure of yolk protein degradation to occur after these proteins enter this modified lysosome compartment, and from their subsequent movement into mature yolk platelets that lack demonstrable lysosomal enzyme activity.

MATERIALS AND METHODS

Animals and Reagents

Collagenase (type IV), hyaluronidase (type III), acid phosphatase (type III), and substrates for the assay of lysosomal enzymes were purchased from Sigma Chemical Co. (St. Louis, MO). Dye reagent concentrate for protein determination was obtained from Bio-Rad Laboratories (Richmond, CA). High purity sucrose for density gradient centrifugation was from Schwarz-Mann (Spring Valley, NY), and glutaraldehyde and OsO_4 were obtained from Polysciences, Inc. (Warrington, PA). All other chemicals used were reagent grade. Sexually mature Xenopus laevis females were purchased from the South African Snake Farm (Fish Hoek, South Africa). Albino mutants were kindly provided by Dr. Robert Tompkins, Department of Biology, Tulane University. Animals were maintained on a diet of beef liver or frog brittle (Nasco Biologicals, Fort Atkinson, WI).

Protein and Enzyme Assays

Protein assays were performed by the Bio-Rad procedure, using bovine gamma-globulin as standard. NAG activity was measured by the method of Hubbard and Cohn (13), acid phosphatase activity by the method of either Bowers et al. (2) or Filburn (10) using p-nitrophenylphosphate as substrate. β-glucuronidase, β-galactosidase, aryl sulfatase, and cathepsin B were all assayed by the protocols given in Bowers, et al. (2). Cathepsin D activity was measured according to Decroly et al. (6). In all cases, 0.1% Triton X-100 was added to the final assay mix, even if this was not specified in the protocol. Succinate dehydrogenase activity was determined at room temperature by the protocol of Earl and Korner (9) using dichlorophenol-indophenol as substrate.

The problem of turbidity caused by high concentrations of the relatively insoluble yolk proteins was effectively eliminated by centrifugation of the assay tubes at 2,000 rpm for 5 min before the absorbance of the supernates was read.

Preparation of Oocytes

Oocytes were freed of follicle cells and other ovarian tissues by enzymatic dissociation or manual dissection. Ovarian fragments containing ~50 large oocytes per piece were manually separated, then placed into a solution containing 5 mg/ml collagenase, 5 mg/ml hyaluronidase in calcium-free OR-2 (29). The tissue was incubated on a rotating platform at room temperature for 3–6 h, depending on the time required for a particular ovary to dissociate. Since the release of oocytes from the tissue took place over 2–3 h, the free oocytes were removed from the dissociation medium at half-hour intervals to ensure that they did not remain in the enzyme solution for longer than necessary to achieve dissociation. After collagenase treatment, oocytes were rinsed with several changes of OR-2 with Ca, and then sorted under a dissecting microscope to remove unhealthy cells that displayed abnormal pigmentation patterns. Oocytes were stored at 4°C until homogenization. No differences have been

observed in the results with storage of oocytes in this manner for periods ranging from 1 to 24 h. Fractionation of large oocytes that have been manually dissected from their follicles, rather than prepared by enzymatic dissociation, also gave equivalent results. Oocytes of different developmental stages were manually separated using the morphological criteria and terminology of Dumont (8).

Preparation and Labeling of VTG

VTG was purified from the serum of estradiol-treated female Xenopus by the procedure of Wiley et al. (33). The ligand was iodinated by the lactoperoxidase-glucose oxidase method using Enzymobeads from Bio-Rad Laboratories according to the method supplied by the manufacturer.

Homogenization and Fractionation Procedures

HOMOGENIZATION PROTOCOLS: Whole ovary homogenates were prepared by cutting the tissue into fragments containing 50 to 100 oocytes each, rinsing the pieces in cold homogenization buffer (0.25 M sucrose, 0.5 mM $MgCl_2$, 1 mM EGTA, 5 mM HEPES, pH 7.4), and homogenizing with 10–12 fast strokes in a Dounce tissue grinder with the loose-fitting pestle. Both frog liver and rat liver homogenate were prepared by mincing the tissue with scissors and then homogenizing with a motor-driven Potter-Elvehjem tissue grinder in the same buffer as above. In all cases, the ratio of tissue volume to homogenate volume was 1:5.

Homogenization procedures for enzymatically dissociated oocytes varied depending on the stage of development of the cells being used. Oocytes of stages I to III were rinsed several times in homogenization buffer, four times the tissue volume of buffer was added, and the cells were homogenized with 10–15 strokes of the tight Dounce pestle. Large oocytes of stages IV to VI were rinsed as above and homogenized at the same tissue/volume ratio with from three to five slow strokes of the loose Dounce pestle.

SUCROSE GRADIENT FRACTIONATION OF OOCYTE HOMOGENATE: 1-ml aliquots of oocyte homogenates were applied to the top of sucrose gradients consisting of a 0.6-ml cushion of 1.27 g/cc sucrose over which a 10.4-ml linear gradient from 1.08 to 1.24 g/cc sucrose was formed. In addition to sucrose, gradient solutions contained the same concentrations of $MgCl_2$, EGTA, and HEPES, pH 7.4, as the homogenization buffer. The gradients were spun at 20,000 rpm for 12–15 h at 4°C in an SW 41 rotor (Beckman Instruments Inc., Palo Alto, CA) 17-drop fractions (~0.35 ml each) were collected from the top of the gradient with an Auto Densi-Flow II fractionator (Buchler Instruments Inc., Fort Lee, NJ). The densities of solutions and of gradient fractions were determined with an Abbé refractometer (Bausch & Lomb Inc., Instruments & Systems, Div., Rochester, NY).

In some experiments, oocyte homogenates were subjected to a brief centrifugation at 200 g_{av} for 10 min to separate yolk platelets from smaller organelles. Homogenates were layered over a 0.5-ml cushion of 1.27 g/cc sucrose to prevent the platelets from pelleting at the bottom, since pelleting and resuspension of this organelle resulted in the release of much lysosomal enzyme activity into a nonsedimentable fraction. The supernate was removed from over the concentrated yellow yolk platelet band, and the platelet layer was collected. Both platelets and supernate were adjusted to a volume of 1 ml with homogenization buffer and then applied to sucrose gradients as described above.

Pulse-Chase Experiments

Groups of 30 manually dissected oocytes were incubated in OR-2 with 0.5 mg/ml VTG (4.9 × 10⁴ cpm/µg) for 30 min at 20°C. After incubation with ¹²⁵I-VTG, oocytes were rinsed three times with OR-2, then incubated as before in a chase medium of OR-2 with 0.5 mg/ml unlabeled VTG for varying times. After the chase, oocytes were rinsed again with three changes of cold OR-2, then three changes of cold homogenization buffer, and homogenized and applied to sucrose gradients as described above.

Histochemical Localization of NAG Activity

Pieces of Xenopus ovary were fixed for 1 h in 1% glutaraldehyde 0.1 M cacodylate, pH 7.4, on ice, then transferred to 1 M sucrose in 0.1 M Tris, pH 7.5, at 4°C overnight. The tissue was then embedded in OCT compound (Miles Laboratories, Naperville, IL) and frozen in isopentane cooled in liquid nitrogen. 10-µm frozen sections were cut on an IEC cryotome (International Equipment Co., Needham Heights, MA) and mounted on chrome–alum subbed slides. The slides were incubated for the demonstration of NAG activity according to the procedure of Hayashi (12), using napthol AS-BI N-acetyl-β-glucosaminide as substrate and hexazonium pararosaniline as the diazo reagent. After incu-

bation in the reaction medium, the slides were rinsed directly in double-distilled water and examined directly without refixation.

Electron Microscopy

FIXATION AND PROCESSING OF SUBCELLULAR FRACTIONS: 50–400 μl of a given gradient fraction were fixed in suspension for 15–20 min on ice in 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4. The samples were then centrifuged at 106,500 g_{av} for 60 min. Pellets were rinsed twice in cacodylate buffer and postfixed for 1 h on ice with 1% OsO_4 in cacodylate, pH 7.4. After dehydration in ethanol, the pellets were embedded in Poly/Bed 812 (Polysciences Inc.) and stained with lead citrate and uranyl acetate after sectioning.

PREPARATION OF WHOLE FOLLICLES AND DISSOCIATED OO-CYTES: In some cases, individual follicles were dissected manually from ovaries and fixed while the oocytes were still surrounded by follicle cells, stroma, and outer ovarian epithelium. In other cases, enzymatically or manually dissected oocytes were fixed without their surrounding follicular layers. Little difference was seen in the quality of preservation obtained by either method. After fixation for 2 h on ice in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, oocytes were rinsed three times for 15 min each with cacodylate buffer, then postfixed on ice for 2 h in 1% OsO_4 in the same buffer. The remainder of the processing was performed in the same manner as for subcellular fractions.

RESULTS

Lysosomal Enzyme Activity of Fully Grown Oocytes

Fully grown collagenase-dissociated oocytes at stages V to VI (8) were first assayed biochemically for the activity of various lysosomal enzymes. The levels of activity found were compared with those seen in various other frog tissues, including whole ovary homogenate, liver, and unfertilized eggs, as well as with activity in rat liver homogenate. The results of this survey are summarized in Table I. Collagenase-dissociated oocytes displayed very high specific activities of NAG, with levels similar to those found in Xenopus liver. Since ~80% of total oocyte protein is stored yolk, these cells appear to be particularly enriched for this enzyme, in agreement with previous reports of Decroly et al. (6). Specific activities of this lysosomal enzyme were similar in dissociated cells, whole ovary homogenate, and unfertilized eggs, which suggested that the bulk of the enzyme was in the oocytes, not in the surrounding somatic tissue. This observation was confirmed by direct histochemical localization of NAG (see below). In addition to glucosaminidase, oocytes had β-glucuronidase, and low levels of β-galactosidase and a protease activity which hydrolyzed denatured hemoglobin. Although the protease resembled cathepsin D in its substrate preferences, as reported earlier (6), the activity was not inhibited by pepstatin A and therefore may be due to some other enzyme. Since levels of

TABLE I. Specific Activity of Lysosomal Enzymes in Xenopus Oocytes and Other Tissues

	Tissue				
Enzyme	Oocytes (st V–VI)	Ovary	Unfer-tilized eggs	Xenopus liver	Rat liver
	μmol product/h per mg protein				
β-N-acetyl glucos-aminidase	1.18	1.70	1.31	1.06	0.43
β-glucuronidase	0.046	0.049	0.04	0.07	0.31
Acid phosphatase	0.046	0.12	0.038	1.4	2.05
Aryl sulfatase	ND	ND	—	ND	0.34
Cathepsin B	ND	ND	—	—	0.003

st, stage. ND, not detectable.

both β-galactosidase and the protease were difficult to measure due to high backgrounds and low activity, we do not consider them reliable lysosome markers in the oocyte. Collagenase-dissociated oocytes appeared negative for aryl sulfatase and cathepsin B activity, and had very low levels of acid phosphatase activity. Although the specific activity of NAG and β-glucuronidase are similar in the liver and oocytes of Xenopus, acid phosphatase levels are at least an order of magnitude lower in oocytes. Low levels of an enzyme such as acid phosphatase may represent bona fide oocyte activity, but the measured values may also result from the presence of a small number of atretic oocytes that have not progressed to the point of obviously abnormal appearance, or from follicle cell contamination of the dissociated cells. The considerably higher specific activity of acid phosphatase in ovary homogenates versus oocytes and unfertilized eggs suggests this might be the case. Low levels of acid phosphatase activity may also result from competitive inhibition of enzyme activity by the phosphorylated yolk proteins in the assay. Nevertheless, unfertilized eggs have the same amount of yolk protein as do fully grown oocytes but substantially less phosphatase activity. Furthermore, the addition of yolk platelet fractions or purified VTG to in vitro assays of acid phosphatase obtained commercially did not lower the measured activity (data not shown). Although we will continue to call NAG-positive organelles "lysosomes" in this paper, the unusual enzymatic composition of these organelles suggests that they may not be "true" lysosomes (see Discussion).

Activity of NAG During Oocyte Development

Since levels of NAG activity were the highest and therefore most readily detectable of the enzymes surveyed, this enzyme was chosen for most of the subsequent analyses of oocyte lysosomes. Measurement of NAG levels in collagenase-dissociated oocytes of different developmental stages revealed steadily increasing activity throughout oocyte growth, with absolute levels of activity in nanomoles per oocyte per hour of 3–4.72 (stage I), 35–75 (stage II), 267–312 (stage III), 375–482 (mid-stage IV), and 529–972 (stage V–VI). This result suggests that oocytes synthesize lysosomal enzymes throughout their development. High levels of hydrolase activity are clearly present at those vitellogenic stages (III to IV) when oocytes accumulate yolk at maximal rates.
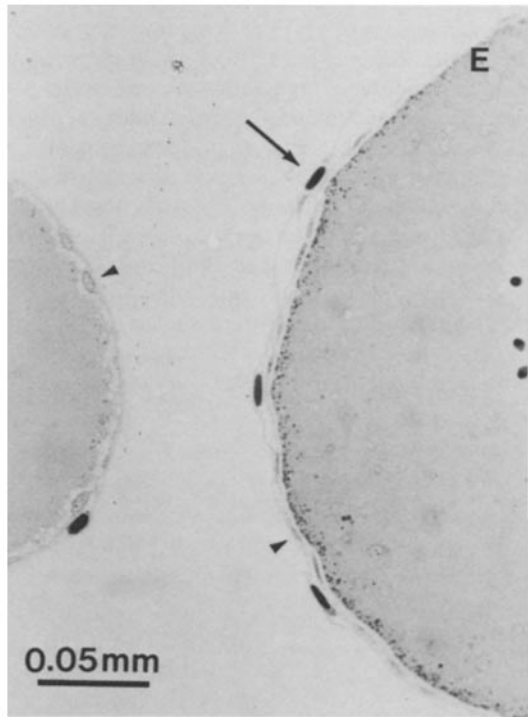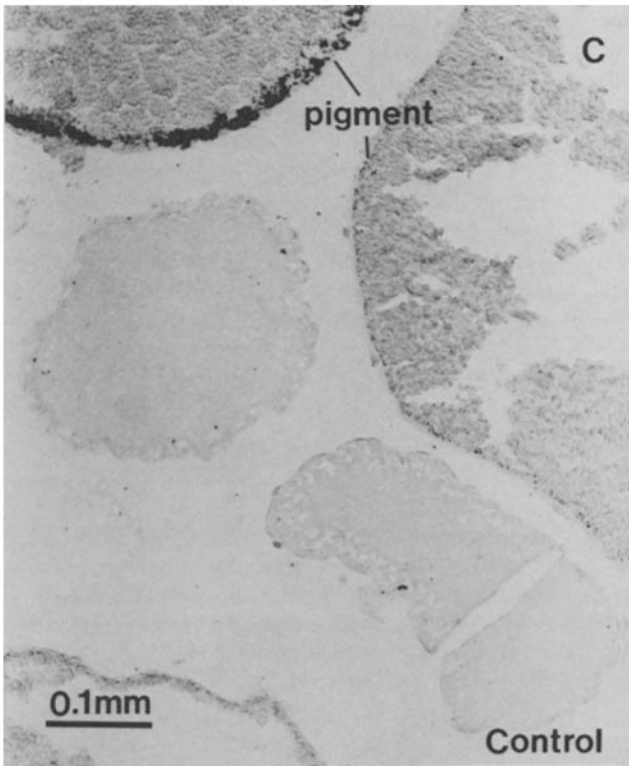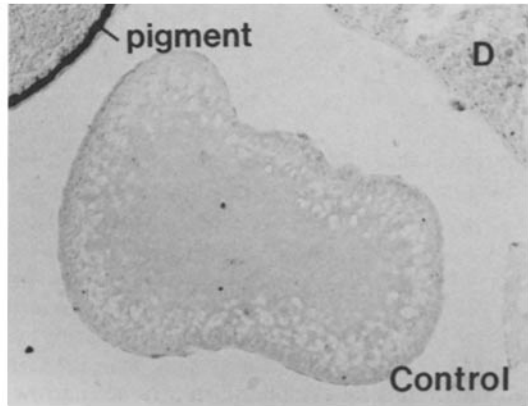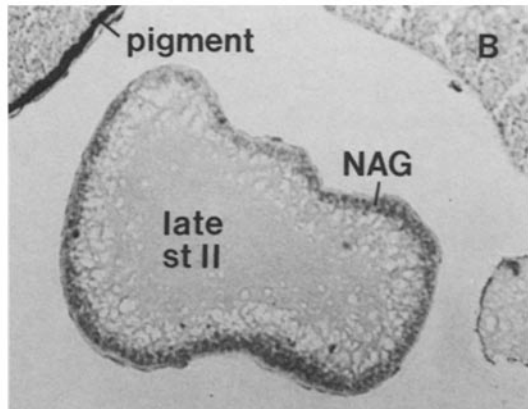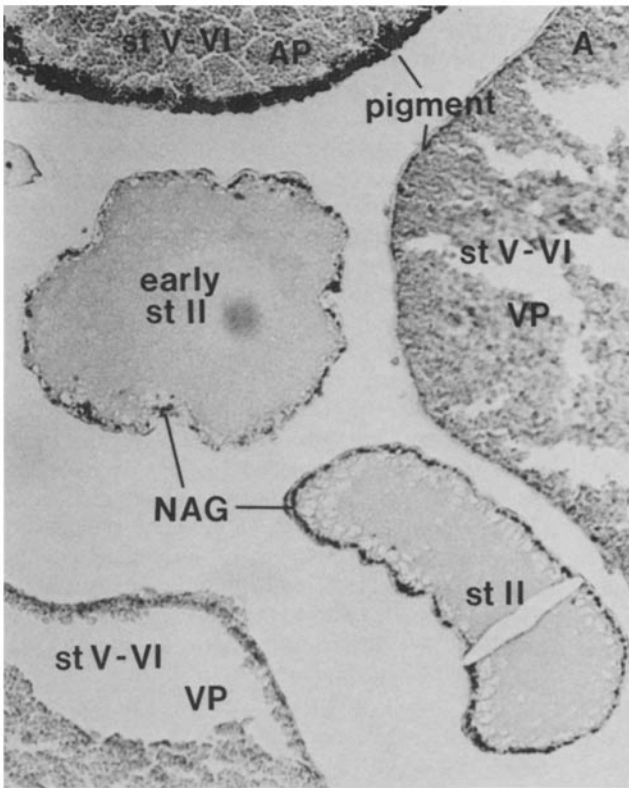
Localization of NAG Activity

The distribution of NAG activity within oocytes at all developmental stages was determined in frozen sections of whole ovary (Fig. 1). In agreement with the biochemical data, the enzyme appeared to be present at all stages of oocyte growth. A striking peripheral distribution was seen, particularly in those cells that appeared to be in the early phases of yolk accumulation. The smallest oocytes had punctate foci of NAG activity at the extreme periphery of the cells (Fig. 1A, early stage II oocyte). As oocyte size increased, the intensity of the NAG reaction product detected per cell at first rose, then decreased somewhat as the reactive zone broadened (Fig. 1, A and B). Occasional small dots of reaction product could be seen in the extremely attenuated follicular epithelium surrounding the oocytes, but the vast majority of activity detected was within the germ cells. Control sections incubated without substrate showed the same light staining of yolk platelets but none of the darker punctate sites of reaction

product deposition (Fig. 1, C and D). Toluidine blue staining of these early vitellogenic oocytes revealed that the distribution of yolk platelets was similar to that of NAG activity (Fig. 1 E). Oocytes in the later stages of growth still appeared to have small peripheral foci of NAG activity, but the sites of activity were not as heavily stained as in earlier stages, and their detection was complicated by a light nonspecific deposition of dye on the accumulated yolk platelets, and by pigment granules at the animal pole of large oocytes.

Ultrastructural Examination of Oocytes

Since previous descriptions of *Xenopus* oocyte morphology were contradictory and did not address the possibility of lysosomes in vitellogenic cells, we next examined oocytes by electron microscopy. This study revealed a number of organelles whose appearance and predominantly peripheral distribution made them likely candidates for the lysosomal compartment seen histochemically (Fig. 2). Stage I previtellogenic oocytes had dense bodies and MVBs that tended to form clusters near the cell surface, which is relatively flat at this stage of development (Fig. 2A). The distance separating clusters of putative lysosomes is quite large. The peripheral cytoplasm of early stage II oocytes also included clusters of MVBs and dense bodies, but the dense bodies were more numerous and had somewhat denser contents (due partly to the beginning of yolk deposition in some of these structures) than did those of the stage I cells (Fig. 2B). The distribution of clusters extends further inside the cell than in earlier oocytes, although they are rarely found more than ~25 μm from the cell membrane. At a slightly later stage, when pigmentation is just barely evident macroscopically, typical yolk platelets are found in the peripheral cytoplasm, in a rather narrow zone extending no more than 45 μm in from the cell membrane. MVBs are still seen in this area, but not always in the clusters typical of earlier stages (Fig. 2C). By the time oocytes have begun to develop obvious pigmentation, the number and size of the yolk platelets have increased, the width of the zone of cytoplasm containing the platelets has broadened, and MVBs and numerous structures with dense contents are found in the peripheral cytoplasm. Although some of the dense bodies superficially resemble those seen at earlier oocyte stages, many of these dense structures represent stages in pigment granule formation, since they are lacking in albino oocytes (not shown). The major feature of oocyte development from this stage to the end of oogenesis is progressive accumulation of yolk platelets until they are the predominant organelle within the cell. MVBs are apparent in the peripheral cytoplasm at all stages in this process.
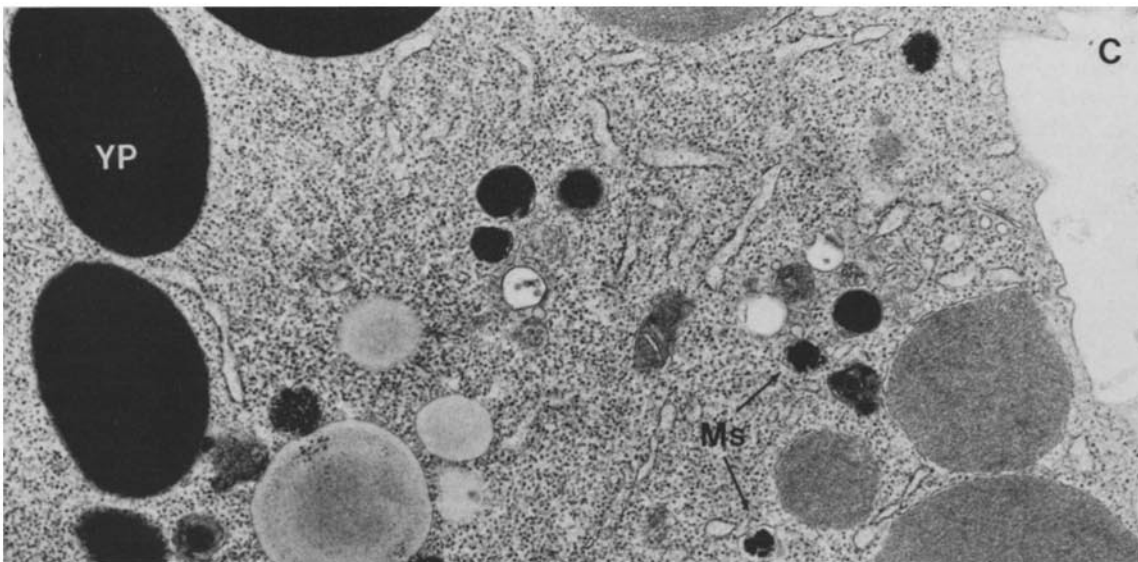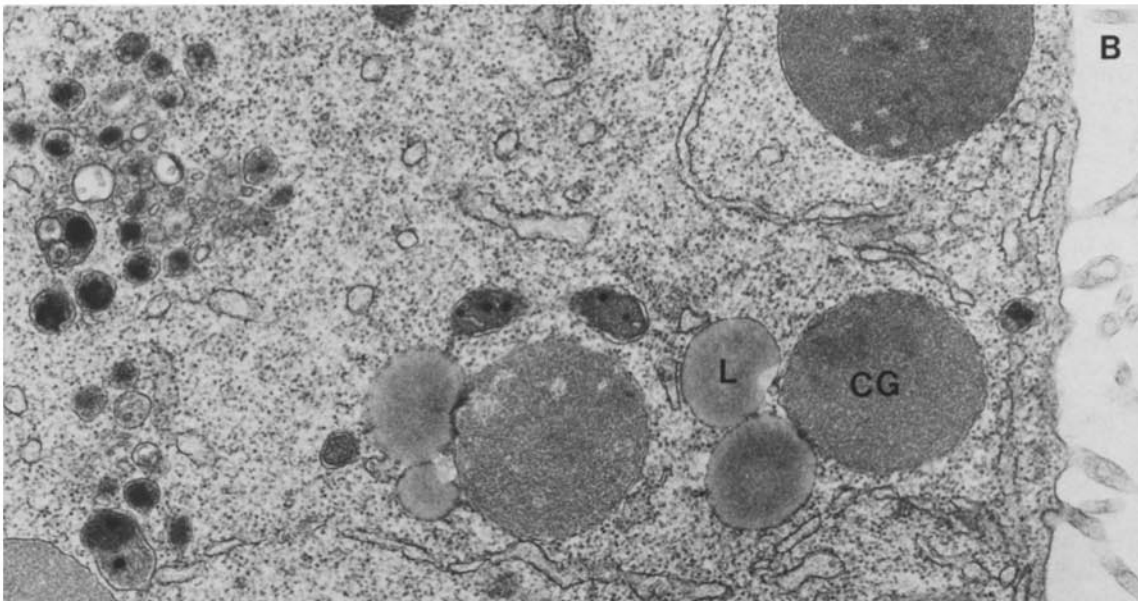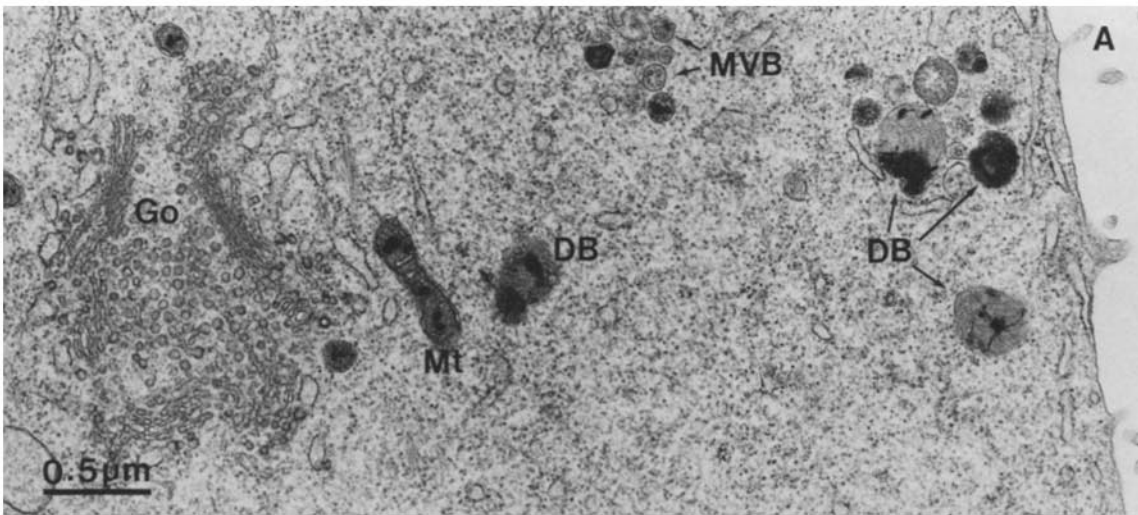
Sucrose Gradient Fractionation of Oocyte Homogenates

Oocytes were next analyzed by subcellular fractionation to gain further information about the distribution of lysosomal enzyme activity. Large oocytes near the end of their growth phase (stage V to VI) were homogenized gently after collagenase dissociation or manual dissection from their follicles, and aliquots were applied directly to sucrose gradients with no preliminary fractionation steps (Fig. 3). After centrifugation, three major bands could be discerned by visual inspection of the gradients. The lowest band, at 1.23 g/cc sucrose, contained most of the protein recovered from the gradient (Fig. 3), and examination of its contents by electron microscopy revealed it to consist of large yolk platelets with crystalline yolk deposits. The middle of the major bands, at 1.21 g/cc sucrose, consisted almost exclusively of yolk platelets virtually identical in appearance to those at 1.23 g/cc, except for a somewhat smaller average size. The sizes of platelets in the two bands overlap considerably, however. The observation of two populations of yolk platelets is in agreement with the earlier findings of Jared et al. (14). The third major band was centered at 1.15-1.16 g/cc and contained predominantly mitochondria, together with a very heterogeneous population of smaller membrane vesicles. The position of this band corresponded to the peak of succinate dehydrogenase activity (data not shown).

Examination of the distribution of NAG across these gradients revealed two peaks of sedimentable activity (Fig. 3). The major peak was at 1.21 g/cc, corresponding closely in position to the light yolk platelet band. Although this region of the gradient contained predominantly yolk platelets, some contamination by smaller membrane vesicles and small dense bodies was present (Fig. 4A). In addition, a second peak of NAG was found in a broad band between 1.12 and 1.18 g/cc. Although this second lysosomal population overlapped considerably with the mitochondria peak, the lysosomal enzyme showed a much wider distribution in the gradient. Examination of the light NAG peak by electron microscopy revealed a very heterogeneous fraction with many mitochondria and various smaller vesicles and tubules, including MVBs (Fig. 4B). The distribution of β-glucuronidase activity was virtually identical to that of NAG (data not shown).

Although two peaks of NAG were always seen on gradients,

FIGURE 1 Histochemical localization of NAG activity in *Xenopus* ovary. 10-μm frozen sections of *Xenopus* ovary fixed in 1% glutaraldehyde were incubated according to the procedure of Hayashi (12) for the demonstration of NAG activity. (A and B) Reaction product is apparent at the periphery of oocytes, particularly in unpigmented cells in very early stages of yolk accumulation (stage II [st II] oocytes in the figure). Little reaction product is found in the extremely thin follicular layer surrounding each oocyte. Although fully grown oocytes (stage V to VI [st V–VI]) show small foci of NAG activity at their periphery, identification of lysosomes is complicated by light nonspecific staining of the yolk platelets that fill their cytoplasm, and by pigment granules concentrated heavily at the animal pole (AP) and scattered at the vegetal pole (VP). × 140. (C and D) Control sections incubated without substrate do not show reaction product at the periphery of early vitellogenic oocytes. × 140. (E) Pieces of ovary fixed and embedded for electron microscopy were sectioned at 1-μm thickness and stained with toluidine blue. This tissue was from an albino female without pigment granules. Dark-staining yolk platelets are accumulating at the periphery of early vitellogenic oocytes, particularly the larger cell at the right. The distribution of yolk platelets and NAG activity are similar in the early phases of yolk deposition. The extreme thinness of the follicle cell layers can be better appreciated in this tissue than in 10-μm frozen sections. Arrowheads point to nuclei of follicle cells, the arrow to an erythrocyte within a follicular capillary. × 290.
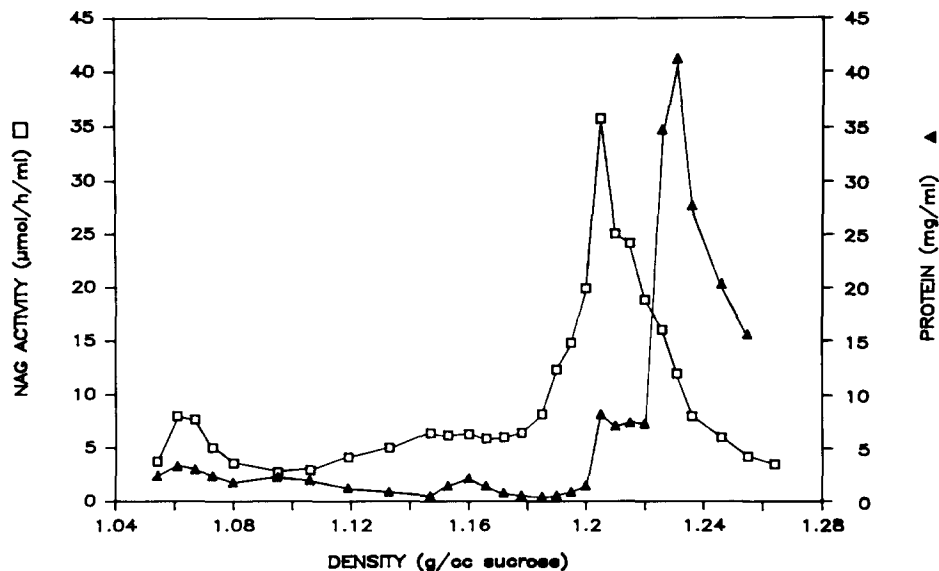
FIGURE 3 Sucrose gradient fractionation of large oocyte homogenates. Oocytes at stages V to VI were freed of follicular tissue by collagenase dissociation and applied to a sucrose gradient for analysis of the NAG and protein distribution. Sedimentable NAG activity was found in a major peak at ~1.21 g/cc, and in a minor, broad peak at 1.12–1.18 g/cc. Protein in fully grown oocytes is seen predominantly in a heavy yolk platelet band at 1.23 g/cc, with a minor band at 1.21 g/cc (light platelet fraction). The position of the major NAG peak coincides with that of the light yolk platelets. Nonsedimentable NAG activity at the left is apparently released by damage of lysosomes during fractionation.

the relative sizes of the two varied somewhat in oocytes obtained from different animals. The two extremes can be seen in Figs. 3 and 5A. The light lysosomal enzyme peak comprised 20–39% of the total sedimentable NAG activity recovered from sucrose gradients. Recovery of NAG activity from the gradient fractions ranged from 73 to 101% in the experiments reported in this paper.

Preliminary Fractionation of the Homogenate Before Gradient Analysis

Although the major peak of NAG activity contained almost pure yolk platelets, some small organelles were also present in this fraction. Therefore, to eliminate the possibility that the enzyme was associated with small structures of the same density as the light platelets, oocyte homogenates were subjected to a preliminary centrifugation at 200 g for 10 min to sediment only very large particles. Both the supernate and the material that sedimented during this spin were then applied to sucrose gradients and fractionated as described above. Another gradient was also run in which an aliquot of homogenate was applied without the 200 g prespin. As seen in Fig. 5, the material sedimenting at 200 g included most of the NAG activity banding at 1.21 g/cc during isopycnic centrifugation. The supernate of the 200 g spin contained the NAG that comprised the broader, light band of activity. Although the presedimentation procedure resulted in considerable damage to lysosomes, with released NAG activity remaining at the top of the gradient, 78% of the total recovered activity remained sedimentable in the experiment shown here. These results suggested that the lysosomal enzyme activity banding at 1.21 g/cc was not contributed by a contaminating small vesicle population and therefore was probably associated with

the yolk platelets themselves. The possibility of adherence of smaller organelles or released enzyme to the platelets seemed unlikely, since the major platelet band at 1.23 g/cc had little associated lysosomal enzyme activity.

Relative Fragility of the Two Lysosomal Compartments

The NAG activity associated with the heavier peak was much more susceptible to damage during homogenization or subsequent manipulations than was the activity associated with the lighter peak. Oocytes from the same animal were homogenized either gently (4 slow strokes of the loose Dounce pestle), or vigorously (12 rapid strokes of the tight pestle). The comparison revealed a substantial loss of activity from the major peak of NAG at 1.21 g/cc sucrose after vigorous homogenization (data not shown). The activity lost was recovered at the top of the gradient as unsedimentable enzyme. Little of the NAG in the light peak appeared to be lost during vigorous homogenization. Susceptibility of the major lysosomal enzyme peak to homogenization damage does not apply to oocytes in early developmental stages, however. The same homogenization conditions that damage heavy lysosomes in large oocytes do not result in significant damage to lysosomes of similar density in small oocytes (Fig. 6). This difference may result from increasing size of the platelets in the 1.21 g/cc fraction as oogenesis proceeds (compare Figs. 4 A and 7 C).

Sucrose Gradient Fractionation of Oocytes in Early Developmental Stages

Since the major NAG peak appeared to be associated with

FIGURE 2 Electron micrographs of the peripheral cytoplasm of oocytes at different developmental stages. Oocytes were fixed while still within their follicles, without exposure to collagenase solutions. (A) Stage I oocyte. Clusters of dense bodies (DB) and MVBs are seen near the cell periphery. A Golgi complex (Go) is also found in this area, as are scattered mitochondria (Mt) and cisternae of rough endoplasmic reticulum. (B) Stage II oocyte. Clusters of dense bodies and MVBs are more prominent than in earlier stages, and the number of dense bodies has increased. Cortical granules (CG) and lipid droplets (L) are now beginning to accumulate near the oocyte periphery. (C) Early stage III oocyte. Typical yolk platelets (YP) are now seen in the peripheral cytoplasm. MVBs are still found at this and all later stages of oocyte development. Pigment deposition in melanosomes (Ms) is beginning to occur. (A–C) × 27,400.
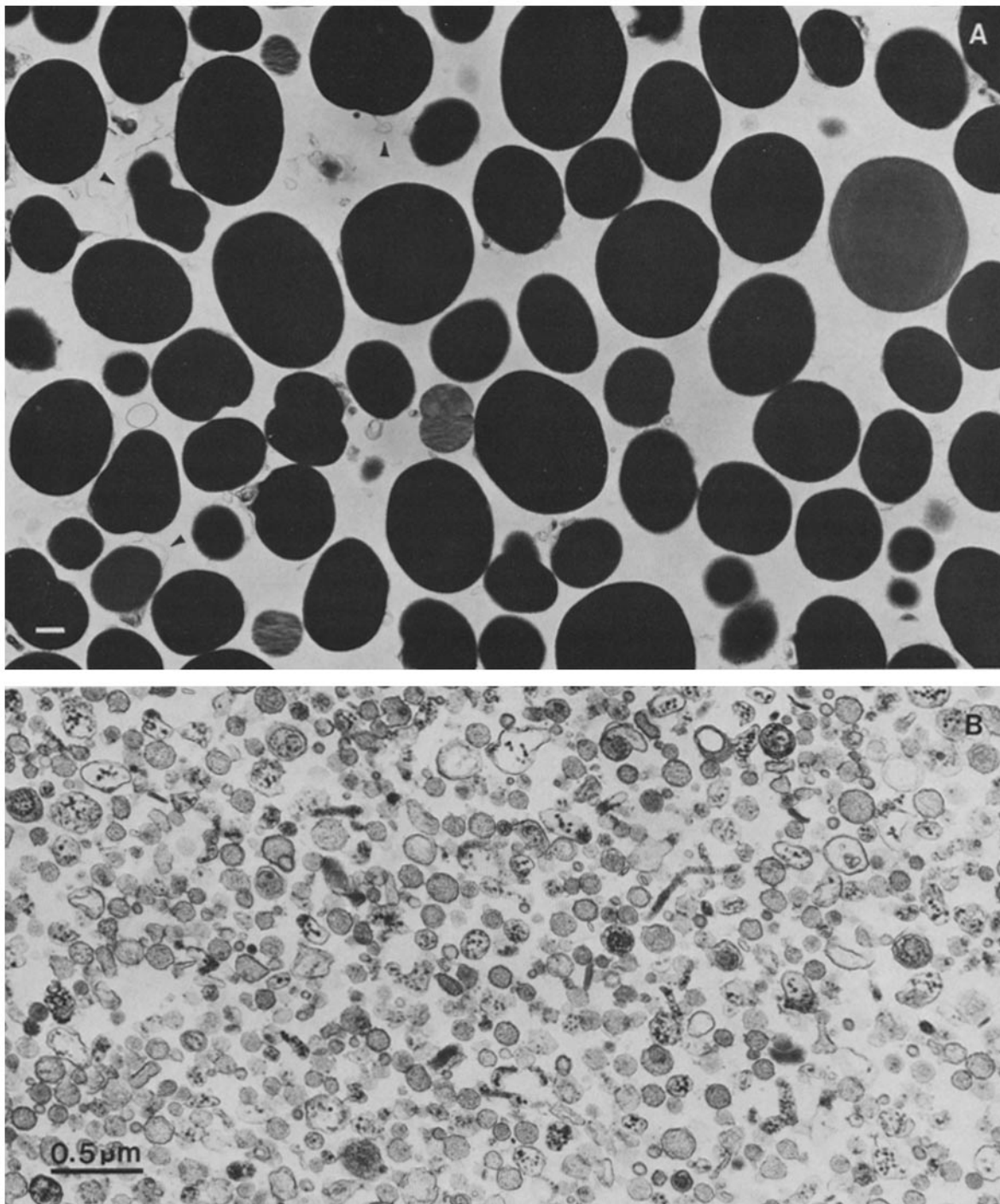
FIGURE 4 Electron micrographs of NAG peak fractions from sucrose gradients. Sucrose gradient fractions containing the peaks of NAG activity were fixed, pelleted, and processed for electron microscopy as described in Materials and Methods. (A) The major peak fraction of NAG activity at 1.21 g/cc sucrose consists predominantly of yolk platelets, although smooth membrane vesicles of uncertain origin and occasional smaller dense structures are also seen. Because yolk platelet membranes often appear damaged, many of the membrane "vesicles" seen here are in fact attached to the platelets (e.g., at arrowheads) and are therefore components of the platelets themselves rather than contaminating organelles. × 8,200. (B) Fractions from the light peak of NAG activity are very heterogeneous in content. Mitochondria are abundant at the bottom of the pellet (not shown). The middle (shown here) and upper regions of the pellet of fixed material consist of smooth membrane vesicles and tubules with a variety of contents. × 28,000.

yolk platelets, we examined oocytes in very early stages of development to determine the NAG distribution when this organelle is first forming within the cells. Examination of the NAG profiles of oocytes just before and after they have entered the vitellogenic phase of development showed that the lysosomal enzyme peak at 1.21 g/cc was absent in previtellogenic (stage I) oocytes, which have not begun to form crystalline yolk platelets (Fig. 6A). NAG activity from stage I
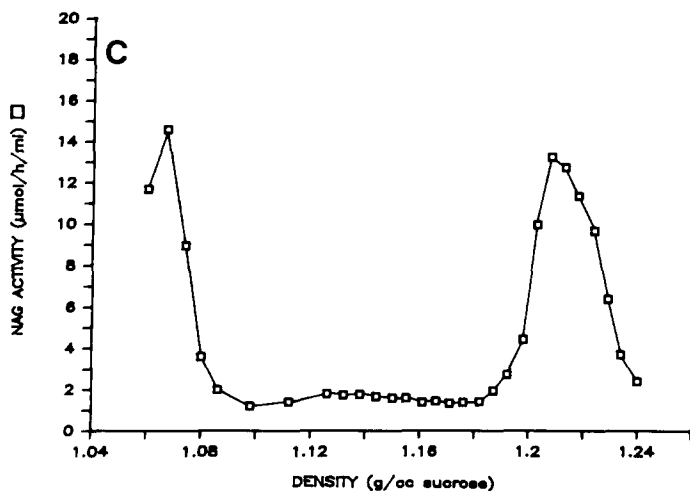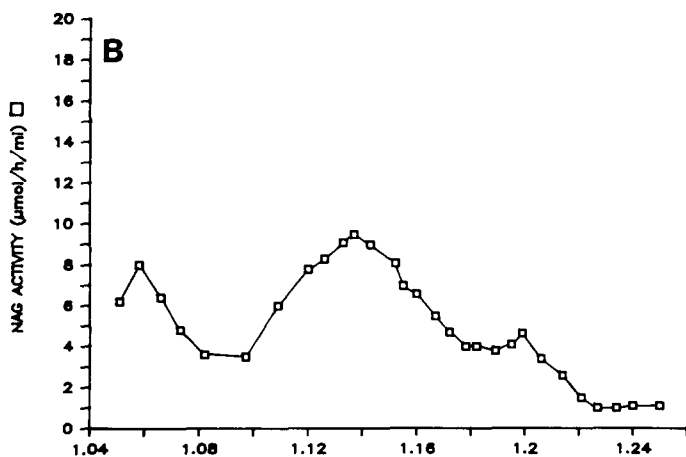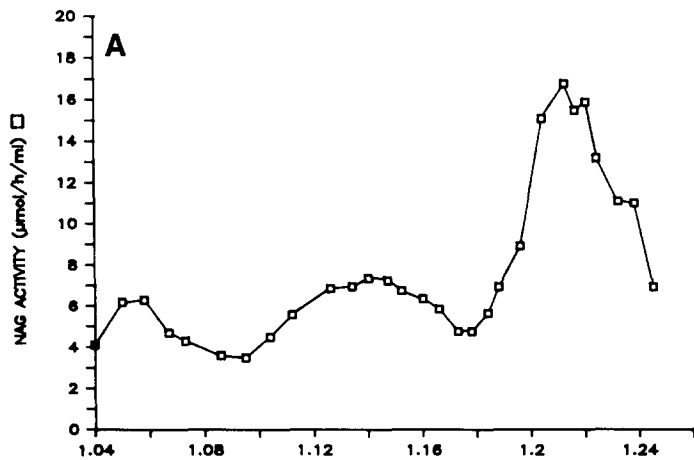
FIGURE 5 The effect of a preliminary sedimentation on the distribution of NAG in sucrose gradients. Stage V to VI oocytes were homogenized and then divided into two 1-ml aliquots. The first was applied directly to a sucrose gradient (A). The second was spun onto a 1.27 g/cc sucrose cushion at 200 g for 10 min, and the supernate (B) and sedimented material from the top of the cushion (C) were both applied to sucrose gradients. Preliminary sedimentation at 200 g eliminated most of the major NAG peak activity at 1.21 g/cc from the gradient profile. In contrast, the organelles contributing to the light lysosome peak remained in the supernate after the preliminary 200 g centrifugation (B). Nonsedimentable activity appearing at the top of the gradients is increased by the preliminary low speed fractionation. This material is probably derived mainly from the heavy lysosome peak, since the lysosomes within this fraction are very susceptible to damage during homogenization and subsequent gradient analysis.

oocytes had a broad profile in sucrose gradients, with a peak density of ~1.18 g/cc sucrose. The peak fraction contained a mixture of membrane vesicles and tubules, granular material, and many mitochondria (at the bottom of the pellet not shown in Fig. 7A). No yolk platelets were seen here or in the 1.21 g/cc region of the gradient (Fig. 7B). Examination of

stage II oocytes revealed the appearance of the major NAG peak at 1.21 g/cc typical of fully grown oocytes. The appearance of this peak coincided with the beginning of yolk protein accumulation at 1.21–1.23 g/cc in the gradient (Fig. 6B) and with the appearance of small yolk platelets in the same gradient fractions (Fig. 7C). In stage III oocytes, the amount
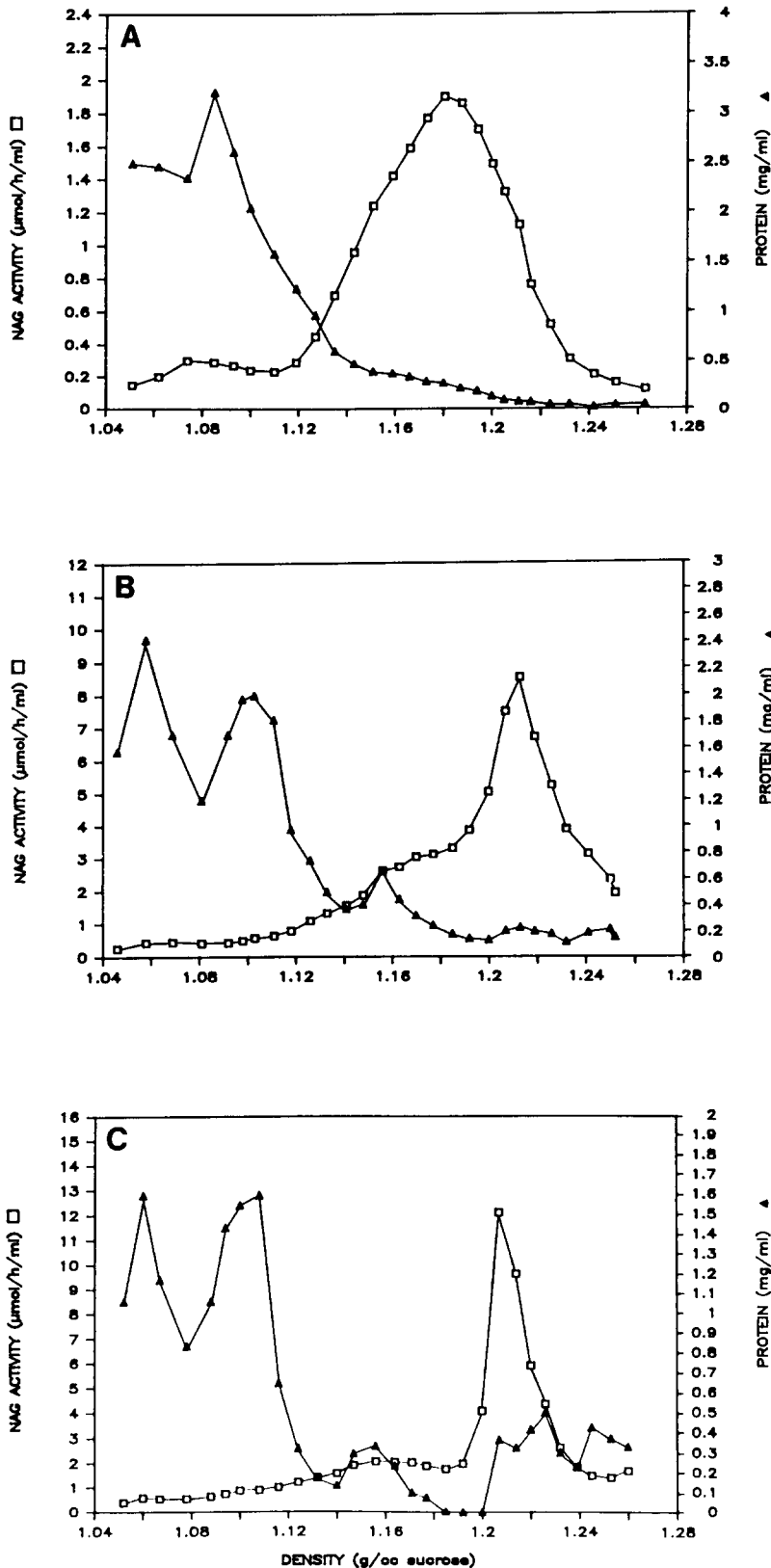
FIGURE 6 Sucrose gradient fractionation of oocytes at early developmental stages. Collagenase-dissociated oocytes were manually separated into different stages. The cells were homogenized as described in Materials and Methods and applied to sucrose gradients. (A) Stage I oocytes. The distribution of NAG activity in the previtellogenic phase of oocyte development is very different from that seen after yolk deposition begins. NAG was found in a single broad peak, ranging from 1.12 to 1.19 g/cc sucrose. No evidence of a peak at 1.21 g/cc is seen. The protein profile obtained from these cells confirms the lack of significant yolk protein accumulation at this time, since little protein is found at 1.21 or 1.23 g/cc in these gradients. (B) Stage II oocytes. As soon as yolk accumulation begins, the NAG activity changes markedly, and a peak of activity at 1.21 g/cc appears. A small amount of protein can now be found in the heavy regions of the gradient. (C) Stage III oocytes. As the process of yolk deposition continues, the pattern of NAG activity typical of fully grown oocytes is established, and increasing amounts of protein are detected in the yolk platelet regions at 1.21 and 1.23 g/cc.

of accumulated yolk protein increased still further, and the pattern of NAG activity distribution typical of fully grown oocytes was established (Fig. 6C). From these results, it appears that the 1.21 g/cc lysosomal compartment is established very early in oocyte development and that its time of appearance coincides with the time of formation of light yolk platelets.

Time Course of ^{125}I-VTG Transport to Yolk Platelets

We examined the movement of iodinated VTG through the endocytic pathway to determine whether the 1.21 g/cc platelet compartment was a normal component of the endocytic pathway to heavy platelets. Fully grown oocytes were

pulse labeled with ^{125}I-VTG for 30 min at 20°C, then chased for varying periods in the presence of unlabeled ligand before homogenization and fractionation on sucrose gradients (Fig. 8). The distribution of ligand after a 45-min chase was quite broad and complex, with a broad peak of activity at 1.20 g/cc and a small amount of ligand at 1.23 g/cc (Fig. 8A). After 1.5 h of incubation with cold VTG, the peak of labeled VTG had sharpened considerably and now appeared at the slightly heavier density of 1.207 g/cc (Fig. 8B). Much less ligand was seen in the lighter regions of the gradient at this time. The small peak at 1.23 g/cc was still present but had not increased significantly since the 45-min chase. However, by 6 h of chase, most of the ligand (~75% by peak weight measurements) had moved to the heavy yolk platelet region at 1.23 g/cc, with the rest remaining at 1.21 g/cc (Fig. 8C). Transport to the heavy yolk platelets therefore appears to involve a relatively slow movement from the light platelet compartment to the final mature platelets at 1.23 g/cc. The significance of the small amount of ligand seen at 1.23 g/cc at the earliest chase times is not currently understood. Although the total counts per minute associated with the oocytes remained quite constant during the chase, a 20–25% increase in trichloroacetic acid–soluble VTG occurred by the end of 6 h, possibly due to degradation or deiodination of ^{125}I-labeled ligand as reported by Opresko et al. (18).

DISCUSSION

Xenopus Oocytes Have Modified Lysosomes Throughout Their Development

The work reported in this paper demonstrates that organelles containing lysosomal hydrolase activity are present in *Xenopus* oocytes throughout their development. The steady rise of NAG activity during oocyte growth indicates that this hydrolase is being synthesized continuously and suggests that new lysosomes are made throughout the time of yolk protein accumulation. The unusual enzyme complement of these lysosomes may be related to the need of the oocyte to accumulate large amounts of protein internalized by receptor-mediated endocytosis, i.e., they may not have the enzymes needed to completely hydrolyze VTG. Since NAG and β-glucuronidase are "typical" components of lysosomes in most eukaryotic cells, we have used the term lysosome for the organelles found in *Xenopus* oocytes. Nevertheless, since different hydrolase activities can be found in discrete populations of subcellular organelles (17), the possibility exists that the unusual lysosomes that we describe here are a distinct orga-
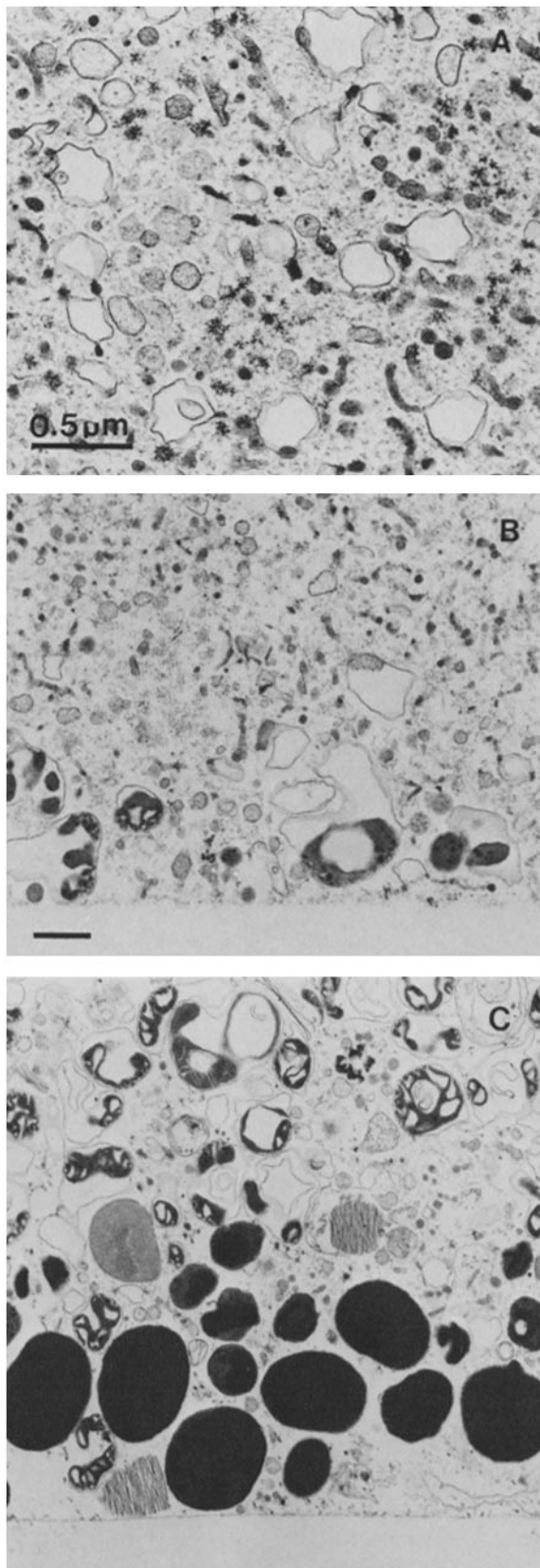
FIGURE 7   Electron micrographs of fractions from sucrose gradients of early stage oocytes. The shift of the NAG peak to 1.21 g/cc correlates with the appearance of yolk platelets in this region of sucrose gradients. Fractions of density 1.18 and 1.21 g/cc were examined after gradient fractionation of oocytes at early developmental stages. (A) The lysosomal peak fraction at 1.18 g/cc from

stage I oocyte gradients contained a heterogeneous mixture of membrane vesicles, tubules, and granular material. Mitochondria were numerous at the bottom of the membrane pellet (not shown here). Fractions of the same density from gradients of stage II oocytes have a similar composition (not shown). × 28,000. (B) Fractions of 1.21 g/cc from the stage I oocyte gradient contained membrane vesicles and tubules, granular material, and damaged mitochondria near the bottom of the membrane pellet. No yolk platelets were seen. × 16,200. (C) In contrast, stage II oocytes, which exhibit an NAG peak at 1.21 g/cc, have yolk platelets in this region of the gradient, as well as the other organelles seen at earlier stages. Although these platelets have a smaller average size than their counterparts at the same density in gradients of fully grown oocytes (compare Fig. 7C with Fig. 4A), they nevertheless exhibit a similar morphology. × 16,200.

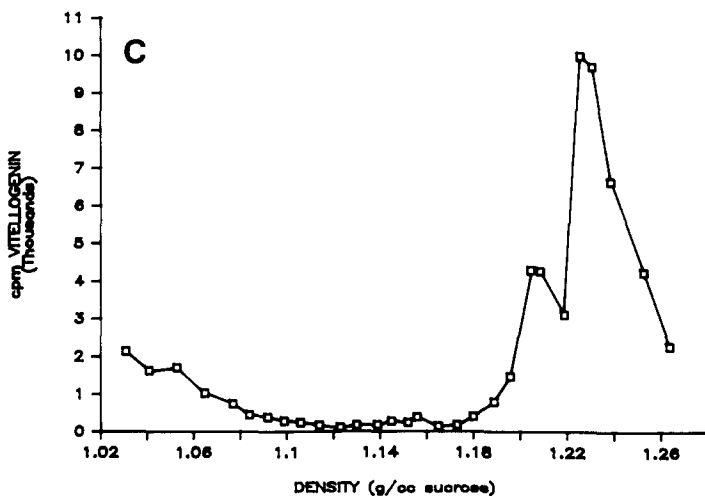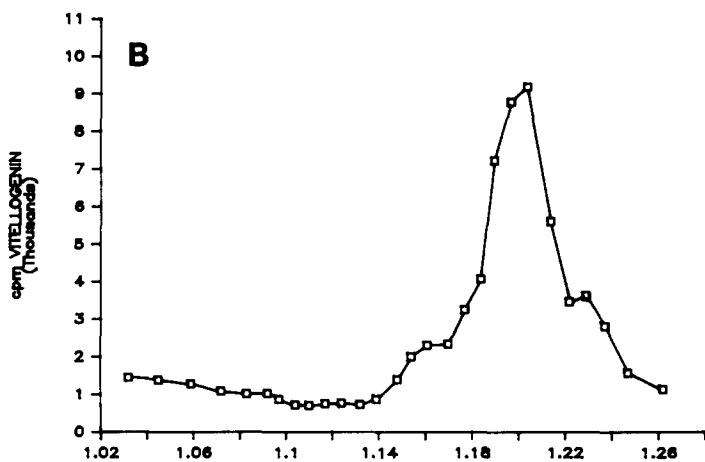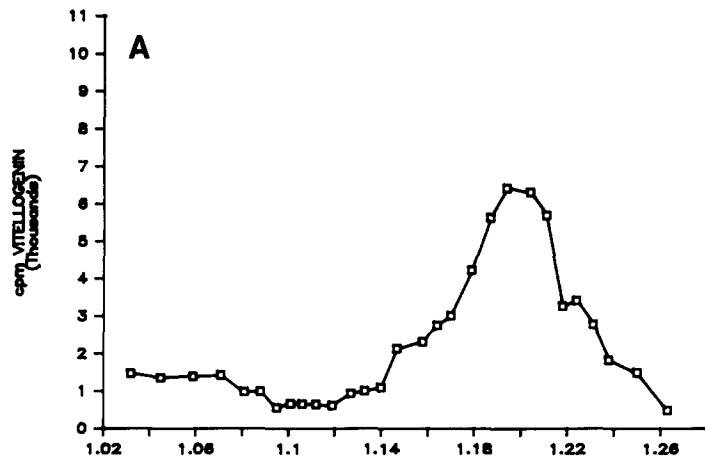FIGURE 8 Sucrose gradient fractionation of oocytes after incubation with ^{125}I-VTG. Groups of 30 fully grown oocytes were manually dissected from their follicles and incubated in 0.5 mg/ml ^{125}I-VTG in OR-2 for 30 min at 20°C. Oocytes were then rinsed, and transferred to OR-2 with 0.5 mg/ml unlabeled VTG for 45 min (A), 1.5 h (B), or 6 h (C) of chase time. (A) After a 45-min chase, the ligand is quite broadly distributed in the gradient, with peak activity at ~1.20 g/cc sucrose and a small peak at 1.23 g/cc. (B) After a 1.5-h chase, a major peak of VTG at 1.207 g/cc is seen, with less ligand in lighter regions of the gradient than seen earlier. The small peak at 1.23 g/cc is still apparent but has not grown appreciably. (C) After a 6-h chase period, most VTG has moved to the heavy yolk platelet position at 1.23 g/cc, although some ligand still remains in a peak at 1.21 g/cc. Total ^{125}I-VTG recovered from each gradient was 66,550 cpm for gradient A, 72,430 cpm for gradient B, and 59,840 cpm for gradient C.

nelle involved, for example, in the processing of VTG after its endocytosis by the cell. True lysosomes may not appear until later, during embryonic development. The high levels of enzymes involved in carbohydrate processing may be involved in modification of VTG, which is a glycoprotein (27). There is not yet direct evidence for such a role, however, since the fate of the carbohydrate moieties of this protein after endocytosis is unknown.

Lysosomal Enzymes Are Associated with a Subpopulation of Yolk Platelets

In this paper we present several lines of evidence that

indicate that most of the lysosomal enzyme activity of *Xenopus* oocytes is found within a population of "light" yolk platelets. These data argue strongly for the presence of both yolk proteins and lysosomal enzymes within the same organelle and suggest that the storage of yolk within the oocyte cannot be attributed to a failure of yolk-containing endosomes to fuse with lysosomes within the oocyte. Direct ultrastructural localization of lysosomal enzyme activity has unfortunately not been possible, since in our studies the *Xenopus* oocyte appears not to have demonstrable levels of those enzymes for which satisfactory histochemical localization techniques are currently available (acid phosphatase, aryl sulfatase, thiolesterase, and trimetaphosphatase have been tried). In this regard, *Xenopus* oocytes are similar to vitellogenic mosquito oocytes, which display only a weak reaction for acid phosphatase in, according to Raikhel, a few autolysosomes, and no activity in yolk-containing bodies (21).

Identity of the Light Lysosome Fraction

The extremely heterogeneous nature of the organelles contained within the light lysosome region at 1.12–1.18 g/cc sucrose precludes certain identification of the organelles that comprise this lysosome compartment. Since unfertilized eggs fractionated on sucrose gradients show the same distribution of NAG activity into light and heavy peaks (data not shown), the activity in the light peak cannot be attributed to a low level of contaminating follicle cells or other ovarian tissues. Three candidates for the light lysosomes suggested by the ultrastructural observations of oocytes are primary lysosomes formed by the Golgi apparatus, MVBs, and dense bodies. Since the levels of NAG rise throughout oocyte development, newly formed primary lysosomes are expected to be present at all times. Previous ultrastructural examination of *Xenopus* oocytes (1) demonstrated MVBs in vitellogenic cells but failed to show these structures in previtellogenic oocytes, in contrast to our observations. Poor fixation of the tissue (no aldehydes were used in tissue preparation) may have been a factor in the failure of these authors to observe MVBs in early oogenesis, when they are less conspicuous. The presence of MVBs in all stages of oocytes and the postulated involvement of these organelles in VTG endocytosis in *Xenopus* and other amphibians (31, 32), as well as in the endocytic pathways in many other cell types (24), suggest that this organelle is another potential contributor to hydrolase activity in the light lysosome peak. Not all MVBs are lysosomes, however, and internalized ligands have been found in multivesicular endosomes that have no demonstrable lysosomal hydrolase activity (e.g., references 11 and 26). In this case, the entrance of endocytosed molecules into multivesicular endosomes appears to occur before fusion with lysosomes and subsequent degradation. An analogous situation may exist for the *Xenopus* oocyte, where the light lysosome peak could include primary lysosomes before fusion with VTG-containing endosomes. After fusion, the density of the lysosome distribution might gradually increase to 1.21 g/cc as a result of continued yolk accumulation (fusion with more VTG-laden endosomes) and cleavage of VTG with subsequent formation of crystalline yolk.

The relatively large percentage of the total yolk found in the light platelet band in stage II and III oocytes (Fig. 6, *B* and *C*) and the absence of significant degradation of VTG during its incorporation by oocytes (18) suggest that the light

platelets are not simply a degradative compartment entered by a small percentage of the total ligand. The reason(s) for the second increase in density of yolk platelets to 1.23 g/cc are not currently known, nor is the mechanism for the apparent loss in enzyme activity during this final transition. Pulse-chase experiments with radiolabeled ligand presented here suggest that the light yolk platelet compartment is a precursor to the heavy platelets, which are the final destination of yolk proteins (Fig. 8; see also reference 19).

Morphological observation of yolk deposition in trout oocytes has revealed the presence of an acid phosphatase–positive MVB compartment in previtellogenic cells (3). As these oocytes enter the vitellogenic phase of their development, internalized vitellogenin appears to accumulate in the MVB compartment, concomitant with a loss of histochemically demonstrable acid phosphatase activity. Although it is not known whether this apparent loss of enzyme activity also occurs for other lysosomal hydrolases, the situation in *Xenopus* oocytes appears to be somewhat different, since a modified lysosome compartment is present throughout oocyte development. In this case, entry of the yolk proteins into the heavy platelet compartment may represent an alternate solution to removing these proteins from continued exposure to hydrolytic enzymes.

Previous work by Decroly, et al. (6) indicated that unfertilized eggs from *Xenopus* contained NAG, protease, and low levels of acid phosphatase activity, but these authors found a very different distribution of enzyme upon cell fractionation than that reported here. Little activity appeared to be associated with yolk platelets, which were sedimented by a low speed centrifugation. However, 60–70% of the total hydrolase activity recovered was released to a nonsedimentable fraction in this work. Based on our experience with the fragility of the lysosome population of density 1.21 g/cc sucrose, the released activity probably came from the major lysosomal enzyme peak of light yolk platelets.

Yolk Degradation During Embryogenesis

Any explanation for the failure of the oocyte to degrade endocytosed VTG must take into account the observation that this protein undergoes complete degradation to amino acids and peptides later during embryonic development. What is missing in the oocyte but present in the embryo? One possible explanation that has been suggested previously (27) and is consistent with the data presented here is that the enzyme complement of the oocyte lysosomes is inadequate to complete the task of VTG cleavage. In this case, the embryo would be required to synthesize the needed enzymes to use its yolk stores. Preliminary studies suggest that the level of at least one of the missing enzymes, acid phosphatase, increases during *Xenopus* embryogenesis (Wall, D., unpublished observations). Acid phosphatase has been shown to be present in the yolk platelets of a number of organisms during yolk breakdown, including the amphibian *Ambystoma* (15), *Drosophila melanogaster* (22), and various marine invertebrates (20). Nevertheless, other explanations are possible, for example, inactivation of lysosomal enzymes still present within the platelets, or raising of the internal pH of the yolk-containing endosomes before or after lysosome fusion (assuming that they are initially acidic endosomes as has been found in other systems). Direct localization of both active and inactive lysosomal enzymes by immunochemical means, as well as deter-

mination of the pH of yolk platelet compartments, will be required to address these possibilities.

REFERENCES

1. Balinsky, B., and R. Devis. 1963. Origin and differentiation of cytoplasmic structures of the oocytes of *Xenopus laevis*. *Acta Embryol. Morphol. Exp.* 6:55–108.
2. Bowers, W. E., J. T. Finkenstaedt, and C. DeDuve. 1967. Lysosomes in lymphoid tissue. I. The measurement of hydrolytic activities in whole homogenates. *J. Cell. Biol.* 32:325–337.
3. Busson-Mabillot, S. 1984. Endosomes transfer yolk proteins to lysosomes in the vitellogenic oocyte of the trout. *Biol. Cell.* 51:53–66.
4. Callen, J. C., M. Tourte, N. Dennebouy, and J. C. Mounolou. 1980. Mitochondrial development in oocytes of *Xenopus laevis. Biol. Cell.* 38:13–18.
5. D'Arcy Hart, P. 1979. Phagosome-lysosome fusion in macrophages: a hinge in the intracellular fate of ingested microorganisms? *In* Lysosomes in Applied Biology and Therapeutics. Vol. 6. J. T. Dingle, P. J. Jacques, and I. H. Shaw, editors. North Holland Publishing Corp., Amsterdam. 409–424.
6. Decroly, M., M. Goldfinger, and N. Six-Tondeur. 1979. Biochemical characterization of lysosomes in unfertilized eggs of *Xenopus laevis. Biochim. Biophys. Acta.* 587:567–578.
7. Dehn, P. F., and R. A. Wallace. 1973. Sequestered and injected vitellogenin: alternative routes of protein processing in *Xenopus* oocytes. *J. Cell Biol.* 58:721–724.
8. Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* 136:153–180.
9. Earl, D. C. N., and A. Korner. 1965. The isolation and properties of cardiac ribosomes and polysomes. *Biochem. J.* 94:721–734.
10. Filburn, C. R. 1973. Acid phosphatase isozymes of *Xenopus laevis* tadpole tails. I. Separation and partial characterization. *Arch. Biochem. Biophys.* 159:683–693.
11. Harding, C., J. Heuser, and P. Stahl. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97:329–339.
12. Hayashi, M. 1965. Histochemical demonstration of *N*-acetyl-β-glucosaminidase employing naphthol AS-BI *N*-acetyl-β-glucosaminide as substrate. *J. Histochem. Cytochem.* 13:355–360.
13. Hubbard, A. L., and Z. A. Cohn. 1975. Externally disposed plasma membrane proteins. II. Metabolic fate of iodinated polypeptides of mouse L cells. *J. Cell. Biol.* 64:461–479.
14. Jared, D. W., J. N. Dumont, and R. A. Wallace. 1973. Distribution of incorporated and synthesized protein among cell fractions of *Xenopus* oocytes. *Dev. Biol.* 35:19–28.
15. Lemanski, L. F., and Aldoroty. 1977. Role of acid phosphatase in the breakdown of yolk platelets in developing amphibian embryos. *J. Morphol.* 153:419–426.
16. McNeil, P. L., L. Tanasugarn, J. B. Meigs, and D. L. Taylor. 1983. Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. *J. Cell Biol.* 97:692–702.
17. Oliver, C. 1983. Characterization of basal lysosomes in exocrine acinar cells. *J. Histochem. Cytochem.* 31:1209–1216.
18. Opresko, L., H. S. Wiley, and R. A. Wallace. 1980. Proteins iodinated by the chloramine T method appear to be degraded at an abnormally rapid rate after endocytosis. *Proc. Natl. Acad. Sci. USA.* 77:1556–1560.
19. Opresko, L., H. S. Wiley, and R. A. Wallace. 1980. Differential postendocytotic compartmentation in *Xenopus* oocytes is mediated by a specifically bound ligand. *Cell.* 22:47–57.
20. Pasteels, J. J. 1973. Yolk and lysosomes. *In* Lysosomes In Biology and Pathology. Vol. 3. J. Dingle, editor. North Holland Publishing Corp., Amsterdam. 216–234.
21. Raikhel, A. S. 1984. The accumulative pathway of vitellogenin in the mosquito oocyte: a high resolution immuno- and cytochemical study. *J. Ultrastruct. Res.* 87:285–302.
22. Sawicki, J.A., and R. J. MacIntyre. 1978. Localization at the ultrastructural level of maternally derived enzyme and determination of the time of paternal gene expression for acid phosphatase-1 in *D. melanogaster. Dev. Biol.* 63:47–58.
23. Steinert, G., and J. Hanocq. 1979. Ultrastructural localization of acid phosphatase activity in matured *Xenopus laevis* oocyte. *Biol. Cell.* 34:247–254.
24. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1–28.
25. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. *Cell.* 28:643–651.
26. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell.* 21:79–93.
27. Wallace, R. A. 1984. Vitellogenesis and oocyte growth: nonmammalian vertebrates. *In* Developmental Biology: A Comprehensive Synthesis. Vol 1. L. Browder, editor. Plenum Publishing Corp., NY.
28. Wallace, R. A., and T. G. Hollinger. 1979. Turnover of endogenous, microinjected, and sequestered protein in *Xenopus* oocytes. *Exp. Cell Res.* 119:277–287.
29. Wallace, R. A., D. W. Jared, J. N. Dumont, and M. W. Sega. 1973. Protein incorporation by isolated amphibian oocytes. III. Optimum incubation conditions. *J. Exp. Zool.* 184:321–333.
30. Wallace, R. A., L. Opresko, H. S. Wiley, and K. Selman. 1983. The oocyte as an endocytotic cell. *Ciba Found. Symp.* 98:228–248.
31. Ward, R. T. 1978. The origin of protein and fatty yolk in *Rana pipiens*. III. Intramitochondrial and primary vesicular yolk formation in frog oocytes. *Tissue & Cell.* 10:515–524.
32. Ward, R. T. 1978. The origin of protein and fatty yolk in *Rana pipiens.* IV. Secondary vesicular yolk formation in frog oocytes. *Tissue & Cell* 10:525–534.
33. Wiley, H. S., L. Opresko, and R. A. Wallace. 1979. New methods for the purification of vertebrate vitellogenin. *Anal. Biochem.* 97:145–152.